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<b>(54) Title:</b> NUCLEOTIDE AND PROTEIN SEQUENCES OF LIVER ACTIVIN/INHIBIN AND METHODS BASED THEREON		
<b>(57) Abstract</b> <p>The present invention relates to the discovery, identification and characterization of a novel subgroup of activins/inhibins, herein referred to as liver activins, that during vertebrate adult life are specifically expressed in the liver. The invention relates to the activin <math>\beta_C</math> and <math>\beta_E</math> subunits and other members of the liver activin subgroup. The invention encompasses liver activin nucleotides; liver activin genomic regulatory elements that regulate the expression of liver activins; host cell expression systems; liver activin proteins, fusion proteins, polypeptides and peptides; antibodies to liver activin protein, transgenic animals that express a liver activin; recombinant knock-out animals that do not express liver activin(s); antagonists and agonists of the liver activins; and other compounds that modulate liver activin gene expression activity to regulate cell growth and/or differentiation and to diagnose and treat abnormalities related thereto. In addition, the present invention encompasses methods and compositions for the regulation of cell growth and/or differentiation including, but not limited to, stimulating liver regeneration, bone growth and hematopoiesis.</p>		

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**NUCLEOTIDE AND PROTEIN SEQUENCES OF  
LIVER ACTIVIN/INHIBIN AND METHODS BASED THEREON**

**1. INTRODUCTION**

5       The present invention relates to the discovery, identification and characterization of a novel subgroup of activins/inhibins, herein referred to as liver activins, that during vertebrate adult life are specifically expressed in the liver. The invention relates to the activin  $\beta_C$  and  $\beta_E$   
10 subunits and other members of the liver activin subgroup. The invention encompasses liver activin nucleotides; liver activin genomic regulatory elements that regulate the expression of liver activins; host cell expression systems; liver activin proteins, fusion proteins, polypeptides and  
15 peptides; antibodies to liver activin protein, transgenic animals that express a liver activin; recombinant knock-out animals that do not express liver activin(s); antagonists and agonists of the liver activins; and other compounds that modulate liver activin gene expression activity to regulate  
20 cell growth and/or differentiation and to diagnose and treat abnormalities related thereto. In addition, the present invention encompasses methods and compositions for the regulation of cell growth and/or differentiation including, but not limited to, stimulating liver regeneration, bone  
25 growth and hematopoiesis.

**2. BACKGROUND OF THE INVENTION**

      The activins and inhibins are dimeric proteins having structural and functional similarity to TGF- $\beta$  superfamily  
30 members. (Vale, W., et al., in Peptide Growth Factors and Their Receptors (Part II) Sporn, M.B. and Roberts, A.B., eds., Springer-Verlag, Heidelberg. pp. 211-248). Like TGF- $\beta$  superfamily members, activin/inhibin is synthesized as a larger precursor that consists of signal peptide, amino-  
35 terminal propeptide and mature growth factor domains. During the synthesis of activin (and other members of the TGF- $\beta$  superfamily), two precursor chains associate to form a

disulfide-bonded dimer with latent activity. The full length dimer is cleaved at an endoproteolytic cleavage motif, which separates the propeptide and mature growth factor domains of the disulfide bonded precursor chains. The propeptide dimer  
5 and the mature growth factor dimer typically remain non-covalently associated. It is believed that the propeptide dimer of activins and other TGF- $\beta$  superfamily members is necessary and sufficient to achieve latency. The mature growth factor domain of activin contains a cysteine residue  
10 motif that is conserved among all known members of the TGF- $\beta$  superfamily (Gray, A.M., et al., 1990, Science 247:1328-1330; Huylebroeck, D., et al., 1990, Mol. Endocrinol. 4:1153-1165; Schwall, R.H., et al., 1988, Mol. Endocrinol. 2:1237-1242). Additionally, the mature growth factor domain of activins  
15 characterized thus far share ~30% amino acid sequence homology with TGF- $\beta$ 1.

Activins/Inhibins have been found to elicit diverse effects in modifying the growth and differentiation of various types of target cells. This complicated regulation  
20 of cellular growth is a characteristic of TGF- $\beta$  superfamily members. For a comprehensive review of TGF- $\beta$  effects, see Roberts, A.B., et al., 1990, in Handbook of Experimental Pharmacology, 95 (Part 1) Sporn, M.B. and Roberts, A.B., eds., Springer-Verlag, Heidelberg. pp. 419-472. The  
25 autocrine and paracrine activities of TGF- $\beta$  are believed to be regulated by different mechanisms. One regulatory strategy involves the temporal and spatial control of TGF- $\beta$  gene expression. A second strategy involves the production and storage of TGF- $\beta$  as a latent complex that is activated  
30 only under certain physiological and pathological conditions, such as, for example, tissue morphogenesis and wound healing. See e.g., Pircher, R., et al., 1986, Biochem. Biophys. Res. Commun. 136:30-37; Miyazono, K., et al., 1988, J. Biol. Chem. 263:6407-6415; Wakefield, L.M., et al., 1988, J. Biol.  
35 Chem. 263:7646-7654). It is likely that activin/inhibin activities are regulated by similar strategies. There is evidence to suggest that activin/inhibin activity is

regulated by association with binding proteins, such as, for example, follistatin, which inhibits activin activity.

Activins and inhibins consist of structurally related polypeptide chains known as  $\alpha$  and  $\beta$  subunits. Presently, two  
5 unique but related  $\beta$  subunits have been identified ( $\beta_A$  and  $\beta_B$ ), which are shared by both inhibin and activin. Consequently, there are three known activins (A, B, and AB) that consist of different combinations of  $\beta$  subunits (e.g.,  $\beta_A\beta_A$ ,  $\beta_B\beta_B$ , and  $\beta_A\beta_B$ ), and there are two known inhibins, each  
10 consisting of one  $\beta$  subunit and one  $\alpha$  subunit ( $\alpha\beta_A$  and  $\alpha\beta_B$ ) (Mason, A.J., et al., 1985, Nature 318:659-663; Mason, A.J., et al., 1986, Biochem. Biophys. Res. Commun. 135:957-964; Forage, R.G., et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:3091-3095; Esch, F., et al., 1987, Mol. Endocrinol. 1:388-  
15 396).

Recently, a cDNA encoding a third activin  $\beta$  subunit, designated activin  $\beta_C$  has been isolated from a human liver cDNA library. Hotten et al., 1995, Biochem. Biophys. Res. Commun., 206:608-613. The product of this clone has yet to  
20 be isolated or recombinantly expressed, and its function is currently unknown. There is ~50% amino acid identity between the conceptualized mature part of human activin  $\beta_C$  and the corresponding regions of human activin  $\beta_A$  and activin  $\beta_B$ ; this is significantly more identity than exists between  $\beta_C$  and  
25 other known members of the TGF- $\beta$  superfamily.

A fourth activin, designated activin  $\beta_D$  was recently isolated from a *Xenopus* liver cDNA library (Oda, S., et al., 1995, Biochem. Biophys. Res. Commun., 210:581-588). Northern analysis indicates that  $\beta_D$  is expressed during early stages of  
30 *Xenopus* embryological development. Microinjection of a synthetic mRNA transcribed from the activin  $\beta_D$  cDNA into *Xenopus* embryo blastomeres led to the formation of a secondary body axis, whereas injection of mRNA into animal pole caps led to mesoderm induction. These activities are  
35 consistent with the known activities of activin  $\beta_A$  and  $\beta_B$  during development.

It has not been determined whether the human activin  $\beta_c$  and Xenopus activin  $\beta_o$  subunits assemble into homodimers, heterodimers, or both. Their recent isolation suggests, however, that the number of combinatorial possibilities for both activin and inhibin may in fact be relatively large.

- Inhibin initially was identified as a gonadal peptide that regulated the release of gonadal hormones from the anterior pituitary-e.g., follicular stimulating hormone or FSH (See e.g., deJong, F.H., et al, 1985, Mol. Cell. Endocrinol. 42:95-103; Dubas, M., et al., 1983, Mol. Cell. Endocrinol. 31:187-198; Channing, C.P., et al., 1985, Proc. Soc. Experim. Biol. Medicine 178, 339-361; Robertson, D.M., Foulds, et al., 1985, Biochem. Biophys. Res. Commun. 126:220-226; Rivier, J., et al., 1985, Proc. 7th Intl. Cong. Endocrinol. 655:1141-1144; Miyamoto, K., et al., 1985, Biochem. Biophys. Res. Commun. 129:396-403; Rivier, J., et al., 1985, Biochem. Biophys. Res. Commun. 133:120-127). The major gonadal sites of inhibin synthesis are Sertoli cells (testis) and granulosa cells (ovary). In the male gonad, inhibin antagonizes spermatogonial DNA synthesis and decreases sperm number. Inhibin synthesis by Sertoli cells fluctuates during spermatogenesis, and inhibin binding to different populations of germ cells has been shown to change during the various stages of spermatogenesis. Consequently, interactions between Sertoli cells and germ cells may be mediated in a paracrine fashion by inhibin. Inhibin (with luteinizing hormone) also stimulates steroid hormone production by Leydig cells in vitro. In the female gonad, inhibin antagonizes oocyte meiosis, increases ovarian follicle number, and stimulates steroidogenesis by thecal cells. In mice of both genders, inhibin appears to be an important negative regulator of gonadal stromal cell proliferation (Matzuk, M.M., et al., 1992, Nature 360:313-319), i.e., inhibin-deficient mice develop mixed or incompletely differentiated gonadal stromal tumors that consist of granulosa cells (female mice) and Sertoli cells (male mice). Finally, inhibin regulates the function of

other tissues besides the gonad and the pituitary (See e.g., Sakai, R., et al., 1992, Biochem. Biophys. Res. Commun. 188:921-926. Inhibin A and B are expressed during development in the placenta and during adult life in the spleen and nervous system. In the placenta inhibin may function as an important regulator of human chorionic gonadotropin.

Activin was also initially identified as a gonadal hypophysiotropic hormone, i.e., activin A ( $\beta_A\beta_A$ ) was originally identified in porcine ovarian fluid as a molecule that stimulates FSH release by pituitary cells (ovarian follicle development and maturation is initiated by FSH secretion from the pituitary) (Vale, W., et al., 1986, Nature 321:776-779; Ling, N., et al., 1986, Nature 321:779-782; Ling, N., et al., 1986, Biochem. Biophys. Res. Commun. 138:1129-1137). Recent evidence indicates that activin A can act on oocytes, granulosa cells, and thecal cells, suggesting that the molecule has an intra-ovarian autocrine paracrine effect. Activin A and B have an extensive anatomical distribution, and they have been implicated to play a role in many biological processes besides ovarian follicle maturation-e.g., pituitary growth hormone and adrenocortical tropic hormone secretion (Sadatsuki, M., et al., 1993, Hum. Reprod. 8:1392-1395), hypothalamic oxytocin secretion (Sawchenko, P.E., et al., 1988, Nature 334:615-617), somatostatin induction (Woodruff, T., et al., 1995, Annu. Rev. Physiol. 57:219-244, lymphocyte proliferation (Hedger, M.P., et al., 1989, Mol. Cell. Endocrinol. 61:133-138), erythropoiesis (EP Publ. No. 210,461, published February 4, 1987; Yu, et al., 1987, Nature 330:765-767; Eto, Y., et al., 1987, Biochem. Biophys. Res. Commun. 142:1095-1103; Broxmeyer, H.E., et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:9052-9056; Murata et al., 1988, Proc. Natl. Acad. Sci. USA., 85:2334-2438; Schwall, R.H., et al., 1991, Meth. Enzymol. 198:340-346; Burton et al., U.S. Patent No. 5,071,834, issued December 10, 1991; Shiozaki, M., et al., 1992, Biochem. Biophys. Res. Commun. 183:273-279; Nishimura,

- M., et al., 1991, Biochem. Biophys. Res. Commun. 181:1042-1047), cardiac morphogenesis (Olson, E. et al., 1996, Science 272:671-672), neuronal survival (Schubert, D., et al., 1990, Nature 344:868-870; Hashimoto, M., et al., 1990, Biochem. Biophys. Res. Commun. 173:193-200), megakaryocyte differentiation (Fugimoto, K. et al., 1991, Biochem Biophys. Res. Commun. 174:1163-1168), spermatogenesis (Mather, J.P., et al., 1990, Endocrinology 127:3206-3214), bone formation (Oue, Y., et al., 1994, Bone 15:361-366; Inoue, S., et al., 1994, Calcif. Tissue Int. 55:395-397; Luyten, F.P., et al., 1994, Exp. Cell Res. 210:224-229; Ogawa, Y., et al., 1992, J. Biol. Chem. 267:14233-14237; Centrella, M., et al., 1991, Mol. Cell. Biol. 11:250-258), pancreatic insulin secretion, patterning events in vertebrate embryos (Wakefield, et al., 1988, J. Biol. Chem. 263:7646-7654; Gentry, L.E., et al., 1987, Molec. Cell Biol. 7:3418-3427; Ogawa, Y., et al., 1992, J. Biol. Chem. 267:2325-2328), reproductive endocrinology (Pircher, R., et al., 1984, Cancer Res. 44:5538-5543; Gentry, L.E., et al., 1988, Molec. Cell Biol. 8:4162-4168), somatic growth (Sadatsuki et al., 1993, Hum. Reprod. 8:1392-1395; Wakefield, L.M., et al., 1989, Growth Factors 1:203-218) and induction of mesoderm during early embryonic development (Smith, J.C., et al., 1990, Nature 345:729-731; van Den Eijnden-Van Raaij, A.J.M., 1990, Nature 345:732-734; Green, J.B.A., et al., 1990, Nature 347:391-394; Thomsen, G., et al., 1990, Cell 63:485-493; Sokol, S., and Melton, D.A., 1991, Nature 351:409-411; Johansson, B.M., and Wiles, M.V., 1995, Mol. Cell. Biol. 15:141-151). Additionally, previous studies have suggested that activin A may function as an autocrine inhibitor of hepatocyte DNA synthesis in vitro (Yasuda, H., et al., 1993, J. Clin. Invest. 92:1491-1496) and an inhibitor of liver regeneration in vivo (Kogure, K. et al., 1995, Gastroenterology 108:1136-1142; and Schwall, R. H., et al., 1993, Hepatology 18:347-356). These studies demonstrate that activin A, synthesis in hepatocytes can be induced by treatment of these cells with either epidermal growth factor or hepatocyte growth factor.

From the data compiled thus far it is clear that activins and inhibins do not act alone in effecting cell and tissue physiology, but rather act in concert with other small regulatory molecules. In this regard, a recent study has  
5 shown that activin A and BMP-4 work synergistically in embryonic development to induce mammalian mesoderm formation and hematopoiesis. While the gonadal protein follistatin has been reported to be a high affinity activin-binding protein, it does not appear to be involved in activin signal  
10 transduction per se.

Erythropoiesis is the production of red cells that occurs continuously throughout the life of vertebrates to offset cell destruction, thereby enabling sufficient numbers of red blood cells to be available in the blood for proper  
15 oxygenation. The generation of red blood cells occurs in the bone marrow and is under the control of the hormone erythropoietin. The amount of erythropoietin in circulating plasma increases during states of reduced oxygen transport in blood plasma. This condition, termed hypoxia, may be caused  
20 by large losses of blood, resulting from, for example, hemorrhaging, anemia, radiation exposure that destroys red blood cells, reduction in oxygen intake resulting from high altitudes, or prolonged unconsciousness.

Dependent on the cause of anemia, many different types  
25 of pharmaceuticals have been employed to relieve anemic conditions, such as, for example, iron preparations (iron-deficiency related anemia), vitamin B<sub>12</sub> and folic acid (malignant anemia), and adrenocortical steroids such as corticoids (hemolytic anemia). Steroid hormones are known to  
30 have powerful erythropoietic stimulating action and are regarded as effective medicines; however, such hormones exhibit strong side effects and are generally undesirable for administration over long time periods.

Erythropoietin has also recently been proposed as an  
35 effective drug for alleviating anemia. U.S. Pat. No. 4,703,008, issued October 27, 1987 describes the recombinant

production of commercially viable quantities of erythropoietin.

Activins A and B have been found to have erythropoietic-stimulating activity. See EP Publ. No. 210,461, published February 4, 1987; Eto, et al, 1987, Biochem. et Biophys. Res. Commun. 142:1095-1103; Murata et. al., 1988, Proc. Natl. Acad. Sci. USA., 85:2334-2438, Yu et al., 1987, Nature 330:765-767, and for activin B see Burton et al., U.S. Patent No:5,071,834, issued December 10, 1991.

10 Activin binding sites have been identified on a number of activin-responsive cells, and chemical crosslinking experiments suggest that multiple binding molecules exist on the cell surface. Additionally, several activin receptors have been isolated and characterized recently. The first of  
15 these to be identified (Mathews, L.S., et al., 1991, Cell 65:973-982) was a 494 amino acid residue integral membrane protein with the characteristics of serine/threonine-specific protein kinase. Since then, a number of TGF- $\beta$  type I and type II signaling receptors have been shown to bind activin  
20 (Attisano, L., et al., Cell 75:671-680; Ebner, R., et al., 1993, Science 262:900-902; ten Dijke, P., et al., Science, 264:101-104).

### 3. SUMMARY OF THE INVENTION

25 The present invention relates to the discovery, identification and characterization of nucleotides that encode a novel subgroup of activins/inhibins, herein referred to as liver activins, and the discovery that these nucleotides correspond to mRNA that during adult life, is  
30 specifically expressed in the liver. More specifically, the invention relates to members of the liver activin subgroup that are defined, herein, as those activins that during adult life are expressed in the liver and demonstrate ~65% identity at the amino acid level in substantial stretches of activins  
35  $\beta_C$  and/or  $\beta_S$ .

Liver activins, like other activin subfamily members, occur naturally as homodimers or heterodimers and regulate

cell growth and/or differentiation through binding to cell surface receptors. The amino acid sequence organization of liver activins is found in other activin family and TGF- $\beta$  superfamily members, and while showing significant homology to activins  $\beta_A$  and  $\beta_B$ , liver activins are most closely related to activin  $\beta_D$ .

For clarity of discussion, the invention is described in the subsections below, by way of example, for activin  $\beta_C$ , depicted in FIG. 1 and activin  $\beta_E$ , depicted in FIG. 2 (murine) and FIG. 5 (human). However, the principles may be analogously applied to other members of the liver activin subgroup.

The activin  $\beta_C$  and activin  $\beta_E$  mRNA transcripts, which are about 2 kb and 3.2 kb long, respectively, are specifically and highly expressed in adult vertebrate liver tissue. The murine activin  $\beta_C$  and  $\beta_E$  cDNAs, described herein, encode proteins of 352 and 350 amino acids, respectively (FIG. 1 and FIG. 2). The human activin  $\beta_E$  encodes a protein of 350 amino acids. Activin  $\beta_C$  and  $\beta_E$  have a N-terminal signal peptide followed by a propeptide region and a mature growth factor region containing nine cysteine residues, the spacing of which is conserved among TGF- $\beta$  superfamily members.

The invention encompasses: (a) nucleotides that encode vertebrate liver activin, including activin  $\beta_C$  and  $\beta_E$  gene products; (b) nucleotides that encode portions of the liver activin that correspond to its functional domains, and the polypeptide products specified by such nucleotide sequences, including, but not limited to, the N-terminal signal peptide, the propeptide domain, and/or the mature growth factor domain of activin  $\beta_C$  and  $\beta_E$ ; (c) nucleotides that encode mutants of the liver activin in which all or a part of one of the domains is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including, but not limited to, nonfunctional proteins in which all or a portion of the N-terminal signal peptide, the propeptide domain, endoproteolytic motif, or mature growth factor domain is deleted; and (d) nucleotides that encode fusion proteins

containing the liver activin or one of its domains (e.g., the mature growth factor region) fused to another polypeptide.

The invention also encompasses genomic fragments containing the murine activin  $\beta_c$  and  $\beta_e$  genes. Several  
5 genomic clones have been isolated and a map of the murine genomic region is shown in Figure 4A. Genomic nucleotide sequences that regulate the expression of the liver activin genes, such as those positioned between the murine activin  $\beta_c$  and  $\beta_e$  genes, are also encompassed by the present invention  
10 (FIG. 4B).

The invention also encompasses agonists and antagonists of liver activins, including small molecules, large molecules, mutant liver activins that compete with native liver activin and antibodies; nucleotide sequences that can  
15 be used to inhibit the liver activin gene expression (e.g., antisense and ribozyme molecules, and gene or regulatory sequence replacement constructs) or to enhance the liver activin gene expression (e.g., expression constructs that place the liver activin gene under the control of a strong  
20 promoter system).

In addition, the invention encompasses transgenic animals that overexpress a liver activin transgene. Such animals may be developed to serve as bioreactors for production of liver activins. The expression of liver  
25 activins via transgenic livestock has the advantage over protein production in recombinant bacteria and yeast in the ability to produce proteins which are correctly post-translationally processed and modified. Transgenic animals may also be developed that do not express liver activin  
30 ("knock-out mice").

In addition, the present invention encompasses methods and compositions for the diagnostic evaluation, typing and prognosis of cell growth and/or differentiation disorders, and for the identification of subjects having a  
35 predisposition to such conditions. For example, liver activin nucleic acids can be used as diagnostic hybridization probes or as primers for diagnostic PCR analysis to identify

liver activin gene mutations, and polymorphisms, variations as well as regulatory defects in the liver activin gene. The present invention further provides for diagnostic kits that allow the practice of such methods.

5       The liver activin protein, polypeptides, and derivatives (including fragments) disclosed herein may be used for the production of anti-liver activin antibodies which may be used diagnostically in immunoassays for the detection or measurement of liver activin protein in a patient sample.

10      Anti-liver activin antibodies may be used, for example, for the diagnostic detection or measurement of liver activin protein in blood serum or biopsied cells and tissues.

          In addition, the present invention encompasses methods and compositions for the regulation of cell growth and/or  
15      differentiation, including, but not limited to, stimulating liver regeneration, bone growth or hematopoiesis.

Therapeutic compounds of the invention include but are not limited to liver activin proteins, polypeptides, and derivatives (including fragments) thereof; antibodies  
20      thereto; nucleic acids encoding the liver activin proteins, polypeptides, and derivatives; and liver activin antisense nucleic acids.

          Further, the present invention also relates to methods for the use of liver activin genes and/or liver activin gene  
25      products to identify compounds which modulate liver activin gene expression and/or liver activin gene product activity, i.e., act as agonists or antagonists. Such compounds can be used as agents to control cell growth and/or differentiation, such as, for example, therapeutic agents that stimulate cell  
30      growth and differentiation, including liver regeneration, hematopoiesis and/or bone growth.

          The present invention also relates to methods of production of the liver activin protein, polypeptides, derivatives and antibodies, such as for example, by  
35      recombinant means.

          This invention is based, in part, on the surprising discovery, of a subgroup of activins that are specifically

and highly expressed in adult vertebrate liver, the identification and cloning of liver activin  $\beta_c$  and  $\beta_e$  cDNAs from libraries prepared from adult vertebrate liver mRNA, characterization of the nucleotide sequence of activin  $\beta_c$  and  $\beta_e$ , expressing activin  $\beta_c$  in a cell-free transcription and translation system and determining that  $\beta_c$  product stimulates liver regeneration, stimulates bone growth in a critical defect model, stimulates hematopoiesis and specifically binds the latent TGF- $\beta$  binding protein-3 (LTBP-3) in vitro.

10

### 3.1 DEFINITIONS

As used herein, the following terms, whether used in the singular or plural, will have the meanings indicated:

Liver activin gene, nucleotides or coding sequences: means nucleotide sequences encoding liver activin/inhibin  $\beta$  subunit protein, polypeptide or derivatives (including fragments) of liver activin/inhibin  $\beta$  subunit protein or liver/activin inhibin  $\beta$  subunit fusion proteins. Liver activin nucleotide sequences encompass liver activin/inhibin DNA, including genomic DNA (e.g. the  $\beta_c$  or  $\beta_e$  gene) or cDNA, or RNA.

Liver activin genomic fragment: means a genomic DNA fragment comprising nucleotide sequences positioned adjacent to, i.e., upstream or downstream, of the liver activin coding regions. Such nucleotide sequences can contain regulatory elements, i.e., promoters, enhances, etc., which regulate the expression of liver activin genes.

Liver activin: means monomers or dimers containing liver activin/inhibin  $\beta$  subunit protein. Polypeptides or derivatives (including fragments) of liver activin/inhibin  $\beta$  subunit protein are referred to as liver activin polypeptides or liver activin derivatives. Fusions of liver activin/inhibin  $\beta$  subunit proteins, or liver activin/inhibin  $\beta$  subunit polypeptides or inhibin  $\beta$  subunit derivatives (including fragments) to an

unrelated protein are referred to herein as liver  
 activin fusion proteins. A functional liver activin  
 refers to a protein which forms an active  
 activin/inhibin monomer, homodimer or heterodimer  
 5 in vivo or in vitro and which is capable of regulating  
 cellular growth and/or differentiation.

#### 4. DESCRIPTION OF THE FIGURES

FIG. 1. The full length mouse activin  $\beta_c$  cDNA sequence.  
 10 The nucleotide sequence of mouse activin  $\beta_c$  is shown, with the  
 predicted amino acid sequence below the coding sequence. A  
 typical signal peptide is predicted to include the first 20  
 amino acid residues. The position of four potential sites of  
 N-linked glycosylation are denoted by a double underline. A  
 15 cluster of basic residues (KHRVRRR, at amino acid residues  
 230-236) may serve as an endoproteolytic cleavage site(s)  
 that defines the predicted propeptide and mature growth  
 factor domains.

FIG. 2. The full length mouse activin  $\beta_e$  cDNA sequence.  
 20 The nucleotide sequence of mouse activin  $\beta_e$  is shown, with the  
 predicted amino acid sequence below the coding sequence. A  
 typical signal peptide is predicted to include the first 21  
 amino acid residues. The position of a single potential site  
 of N-linked glycosylation is denoted by a double underline.  
 25 A cluster of basic residues (RARRR, amino acid residues 232-  
 236) may serve as an endoproteolytic cleavage site (or sites)  
 that defines the predicted propeptide and mature growth  
 factor domains.

FIG. 3. Comparison of the predicted mouse activin  $\beta_c$   
 30 and  $\beta_e$  amino acid sequences with other activin group members.  
 Alignment of the C-terminal acid sequence of mouse activin  $\beta_c$   
 and  $\beta_e$  (beginning at amino acid 236 in both instances) with  
 corresponding regions of human activin  $\beta_A$ , human activin  $\beta_B$ ,  
 and *Xenopus* activin  $\beta_D$ . The nine invariant cysteines  
 35 characteristic of the activins are shown in bold. Dashes  
 denote gaps introduced to maximize the alignment. Species

are indicated as follows: h, human; m, mouse; and x, *Xenopus*.

FIG. 4A. A map representing the genomic organization of the mouse activin  $\beta_c$  and mouse activin  $\beta_e$  genes. For both 5 loci, the 5' and 3' untranslated regions are shown as black rectangles, sequences that encode the predicted propeptide domains are shown as open boxes, and sequences that encode the predicted mature growth factor regions are shown as patterned boxes. Partial restriction analysis and partial DNA sequence analysis of overlapping genomic clones indicates that the size of the intron separating activin  $\beta_c$  exon 1 and 2 is ~12-kbp. In contrast, DNA sequencing has shown that the size of the intron separating activin  $\beta_e$  exon 1 and 2 is 234-bp.

15 FIG. 4B and 4C. Nucleotide sequence of the genomic region located between the murine activin  $\beta_c$  and  $\beta_e$  genes.

FIG. 4D, 4E, 4F and 4G. Sequence of the mouse activin genetic loci. The sequence includes nucleotide sequences upstream of the activin  $\beta_c$  gene, activin  $\beta_c$ /exon 1, partial 20 sequence of activin  $\beta_c$ /intron 1, activin  $\beta_c$ /exon 2, the activin  $\beta_e$  regulatory region, activin  $\beta_e$ /exon 1, activin  $\beta_e$ /intron 1, activin  $\beta_e$ /exon 2, and the sequence located 3' of the activin  $\beta_e$  gene.

FIG. 5. The full length human activin  $\beta_e$  cDNA sequence. 25 The nucleotide sequence of human activin  $\beta_e$  is shown, with the predicted amino acid sequence below the coding sequence. A typical signal peptide is predicted to include the first 17 amino acid residues. The position of a single potential site of N-linked glycosylation is denoted by a double underline. 30 A cluster of basic residues (RARRR, amino acid residues 232-236 may serve as an endoproteolytic cleavage site (or sites) that defines the predicted propeptide and mature growth factor domains (an arrow separates the two predicted domains). Conserved cysteines in the predicted mature growth 35 factor region are shown in bold type.

FIG. 6. An adult mouse multiple tissue Northern blot (Clontech) as evaluated using specific activin  $\beta_c$  and  $\beta_e$  cDNA

probes (top and middle panels, respectively). RNA samples were loaded as follows: lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; and lane 8, testis. RNA size was determined by the manufacturer using RNA standards (9.5, 7.5, 4.4, 2.4, and 1.35 kb). Relative RNA loading was evaluated by hybridization with a glucose 6-dehydrogenase cDNA probe (bottom panel).

FIG. 7. Expression of the full length mouse activin  $\beta_c$  plasmid expression construct in a cell-free transcription and translation system demonstrated by immunoprecipitation of  $\beta_c$ .

FIG. 8. Evidence that mouse activin  $\beta_c$  stimulates bone growth in a critical defect animal model. Microscopic focus of bone (arrows) that formed following direct in vivo gene transfer using a gene activated matrix.

FIG. 9. Evidence that mouse activin  $\beta_c$  promotes hematopoiesis of human bone marrow cultures in vitro.

## 5. DETAILED DESCRIPTION OF THE INVENTION

Liver activins/inhibins, described for the first time herein, comprise an activin/inhibin subgroup during vertebrate adult life are specifically and highly expressed in liver. Liver activins, like other activin group members, occur naturally as homodimers or heterodimers which regulate cell growth and/or differentiation through binding to cell surface signaling receptors. The amino acid sequence organization of liver activins is similar to that found in other activins and TGF- $\beta$  superfamily members and consists of an N-terminal signal peptide that is followed by a propeptide region and a mature growth factor region containing nine cysteine residues, the spacing of which is conserved among TGF- $\beta$  superfamily members. A endoproteolytic cleavage motif separates the propeptide and mature growth factor domains. While showing significant homology to activins  $\beta_A$  and  $\beta_B$ , liver activins are most closely related to activin  $\beta_D$ .

The invention encompasses the use of liver activin genes and gene products, both transcriptional and translational

including, but not limited to, DNA, mRNA, antisense RNA, ribozymes and protein. The invention further encompasses the use of liver activin polypeptides, and derivatives; antibodies to liver activins (which can, for example, act as  
5 liver activin agonists or antagonists); and antagonists that inhibit activin activity or expression, or agonists that activate activin activity or increase its expression in the diagnosis, treatment, and/or regulation of cell growth and/or differentiation, including, but not limited to stimulating  
10 liver regeneration and/or bone growth in vertebrates, including humans. In addition, liver activin nucleotides and liver activin proteins are useful for the identification of compounds effective in regulating cell growth and/or differentiation.

15 The invention also encompasses nucleotide sequences that regulate liver activin gene expression such as those found adjacent to liver activin genes in the genome. Such nucleotide sequences can be used, for example, in expression vectors to promote liver specific expression of either  
20 activin genes, or any gene of interest for which liver specific expression is desired.

In particular, the invention described in the subsections below encompasses liver activin proteins, polypeptides and derivatives thereof corresponding to  
25 functional domains of the liver activin (e.g., N-terminal signal peptide, propeptide, and/or mature growth factor domains); mutated, truncated or deleted liver activins (e.g. a liver activin with one or more functional domains or portions thereof deleted, such as the propeptide domain,  
30 endoproteolytic cleavage motif and/or the mature growth factor domain); liver activin fusion proteins (e.g. a liver activin or a functional domain of a liver activin, such as the mature growth factor domain, fused to an unrelated protein or peptide such as an immunoglobulin constant region,  
35 i.e., IgFc); nucleotide sequences encoding such products; and host cell expression systems that can produce such liver activin products. The invention also encompasses antibodies

and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of the liver activin, as well as compounds or nucleotide constructs that inhibit expression of the liver activin gene (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote expression of liver activin (e.g., expression constructs in which liver activin coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, etc.). The invention also relates to host cells and animals genetically engineered to express the liver activin (or mutants thereof) or to inhibit or "knock-out" expression of the animal's endogenous liver activin. Transgenic animals overexpressing a liver activin transgene may be developed to serve as bioreactors for production of activin.

The liver activin proteins, polypeptides or derivatives; liver activin fusion proteins; liver activin nucleotide sequences; antibodies; antagonists; and agonists can be useful when detecting mutant liver activin or inappropriately expressed liver activin for the diagnosis of cell growth and differentiation disorders. The liver activin proteins, polypeptides or derivatives; liver activin fusion proteins; liver activin nucleotide sequences; host cell expression systems; antibodies; antagonists; agonists; and genetically engineered cells and animals can also be used to screen for drugs that regulate cell growth and differentiation and/or treatment of disorders related thereto.

Finally, the liver activin protein products (especially derivatives such as peptides corresponding to the mature growth factor domain) and fusion protein products (especially liver activin fusion proteins, i.e., fusions of the liver activin or a domain of the liver activin, e.g., the mature growth factor region to an IgFc), antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists can all be used to regulate cell growth and/or differentiation and/or therapy of disorders related thereto.

For example, the administration of an effective amount of an inactive liver activin (e.g., one in which the endoproteolytic motif has been modified so as to prevent formation of polypeptide corresponding to the mature growth factor) or an anti-idiotypic antibody (or its Fab) that mimics a liver activin would bind to and occupy liver activin receptors, thereby reducing activin receptor availability to the active forms and prevent or reduce the signal cascade which ultimately impacts upon cellular growth and/or differentiation. Nucleotide constructs encoding such liver activin products can be used to genetically engineer host cells to express such liver activin products in vivo; these genetically engineered cells function as "bioreactors" in the body, delivering a continuous supply of liver activin protein, polypeptide or derivative that will bind to and occupy activin receptor sites. Nucleotide constructs encoding functional liver activin protein, polypeptide or derivative; mutant liver activins; and antisense and ribozyme molecules can all be used in "gene therapy" approaches for the modulation of liver activin expression and/or activity in the regulation of cell growth and differentiation and/or treatment of disorders related thereto. Studies described herein demonstrate that  $\beta_c$  product stimulates liver regeneration, bone growth in a critical defect animal model and hematopoiesis. Such studies indicate that the liver activins may be useful both as a cytokine for regulation of cell growth and differentiation and/or as a hormone for regulation of physiological process such as, for example, hematopoiesis and bone growth. Additionally, these studies demonstrate that  $\beta_c$  specifically binds the latent TGF- $\beta$  binding protein-3 (LTBP-3) in vitro. Thus, the invention also encompasses pharmaceutical formulations and methods for regulating cell growth and differentiation and/or treating disorders related thereto.

35        This invention is based, in part, on the surprising discovery of a subgroup of activins that during vertebrate adult life are specifically and highly expressed in liver.

This discovery was made possible using synthesized degenerate oligonucleotide primers based on conserved coding sequences in TGF- $\beta$  superfamily members, bone morphogenetic protein-2 and -4, to amplify mouse genomic DNA and cDNA. The products  
5 of these PCR reactions were cloned, labeled and used as probes to screen a mouse liver cDNA library. One of these probes hybridized to a series of overlapping cDNA clones that, in composite, encode a protein homologous to activin members of the TGF- $\beta$  superfamily. This gene has been  
10 designated activin  $\beta_c$ . Northern blot analysis of multiple adult tissues indicates that activin  $\beta_c$  is highly and specifically expressed in the liver as a transcript of approximately 2.0 kb (See FIG. 6). The gene encoding activin  $\beta_c$  was cloned into an expression vector, and this construct  
15 was then expressed in a cell-free transcription and translation system.

While cloning the mouse activin  $\beta_c$  genomic locus, a genomic sequence located approximately 5.0 kb downstream of the activin  $\beta_c$  gene was observed to hybridize under low  
20 stringency conditions to the probe corresponding to activin  $\beta_c$  cDNA. This genomic sequence was subcloned, labeled and used as a probe to screen a mouse liver cDNA library under highly stringent conditions. Sequence analysis of a composite of overlapping cDNA clones obtained from this screen indicate  
25 that they correspond to a gene encoding a novel activin  $\beta$  subunit that shows strong sequence homology to activin  $\beta_c$ ; this gene has been designated activin  $\beta_e$ . Northern blot analysis of multiple adult tissues indicates that activin  $\beta_e$  is highly and specifically expressed in the liver as a  
30 transcript of approximately 3.2 kb (See FIG. 6).

The mouse activin  $\beta_e$  cDNA was labeled and used as a probe to screen an adult human liver cDNA library. The probe hybridized to two overlapping cDNA clones. Sequence analysis revealed a predicted open reading frame of 1050 nucleotides  
35 coding for a protein with an estimated molecular weight of 39 kD and one potential N-linked glycosylation site. Sequence comparison analysis indicates that the protein is

homologous to murine activin  $\beta_E$ . The complete nucleotide sequence and deduced amino acid sequence of the human activin  $\beta_E$  is depicted in FIG. 5.

Various aspects of the invention are described in 5 greater detail in the subsections below.

### 5.1 THE LIVER ACTIVIN GENE

The liver activin nucleotide sequences of the invention include: (a) the DNA sequences shown in FIG. 1, FIG. 2 and 10 FIG. 5 or contained in the cDNA clones pbC, pbE or pbHE within *E. coli* strain DH5 $\alpha$  as deposited with the American Type Culture Collection (ATCC); (b) nucleotide sequences that encode the amino acid sequences shown in FIG. 1, FIG. 2 and FIG. 5, or the liver activin amino acid sequences encoded by 15 the cDNA clones pbC, pbE or pbHE as deposited with the ATCC; (c) any nucleotide sequence that hybridizes to the complement of the DNA sequence shown in FIG. 1, FIG. 2 and FIG. 5 or contained in the cDNA clones pbC, pbE or pbHE as deposited with the ATCC under highly stringent conditions, e.g., 20 hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New 25 York, at p. 2.10.3) and encodes a functionally equivalent gene product; and (d) any nucleotide sequence that hybridizes to the complement of the DNA sequences that encode the amino acid sequence shown in FIG. 1, FIG. 2 and FIG. 5 or contained in cDNA clones pbC, pbE or pbHE as deposited with the ATCC 30 under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, supra), yet which still encodes a functionally equivalent liver activin gene product. Functional equivalents of liver activin include naturally 35 occurring liver activins present in the same or other species, and mutant liver activins, whether naturally

occurring or engineered. The invention also includes degenerate variants of sequences (a) through (d).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are  
5 therefore the complements of, the nucleotide sequences (a) through (d) in the preceding paragraph. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides  
10 ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may encode or act as liver activin antisense  
15 molecules, useful, for example, in gene regulation (for and/or as antisense primers in amplification reactions of liver activin nucleic acid sequences). With respect to liver activin regulation, such techniques can be used to regulate, for example, cell growth and/or differentiation, including,  
20 but not limited to, stimulation of liver regeneration and/or bone growth. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for liver activin gene regulation. Still further, such molecules may be used as components of diagnostic methods whereby, for  
25 example, the presence of a particular liver activin allele responsible for causing a cell growth and/or differentiation disorder may be detected.

In addition to the liver activin nucleotide sequences described above, full length liver activin cDNA or gene  
30 sequences present in the same species, and/or homologs of the liver activin gene present in other species, can be identified and readily isolated, without undue experimentation, by molecular biological techniques well known in the art. The identification of homologs of liver  
35 activins in related species can be useful for developing animal model systems more closely related to humans for purposes of drug discovery. cDNA libraries or genomic DNA

libraries derived from the organism of interest can be screened by hybridization using the nucleotides described herein as hybridization or amplification probes. For example, the liver activin nucleotide sequences described  
5 above may be used as probes to identify and isolate additional human liver activin homologs from genomic DNA isolated from human cells and/or from cDNA generated from human cells or tissue known, or suspected to express the liver activin gene, such as for example, adult liver tissue,  
10 or ATCC human liver cell lines 7004, 7120, 7128, 7137, 7154, 7274, 7284, 7290, 7297, 7503, 7705, 7821, 7837, 7859 or 7864. Furthermore, genes at other genetic loci within the genome that encode proteins which have extensive homology to one or more domains of the liver activin gene product can also be  
15 identified via similar techniques. In the case of cDNA libraries, such screening techniques can identify clones derived from alternatively spliced transcripts in the same or different species.

Screening can be performed by filter hybridization using  
20 duplicate filters. The labeled probe can contain at least 15-30 base pairs of the liver activin nucleotide sequence, as shown in FIG. 1, FIG. 2 and FIG. 5. The hybridization washing conditions used should be of a lower stringency when the cDNA library is derived from an organism different from  
25 the type of organism from which the labeled sequence was derived. With respect to the cloning of a human liver activin homolog, using murine liver activin probes, for example, hybridization can, for example, be performed at 65°C overnight in Church's buffer (7% SDS, 250 mM NaHPO<sub>4</sub>, 2μM EDTA,  
30 1% BSA). Washes can be done with 2XSSC, 0.1% SDS at 65°C and then at 0.1XSSC, 0.1% SDS at 65°C.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled  
35 sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press,

N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, the labeled liver activin nucleotide  
5 probe may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions. The identification and characterization of human genomic clones is helpful for designing diagnostic tests and clinical protocols for  
10 regulating cell growth and/or differentiation and/or treating cell growth and/or differentiation disorders in human patients. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to  
15 detect mutations within the exons, introns, splice sites (e.g. splice acceptor and/or donor sites), etc., that can be used in diagnostics.

Further, other liver activins and other gene homologs may be isolated from nucleic acids of the organism of  
20 interest by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of amino acid sequences within the liver activin gene product disclosed herein. Such primers may be used to amplify sequences of interest from any RNA or DNA source, preferably  
25 a cDNA library. PCR can be carried out, e.g., by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp<sup>®</sup>). The DNA being amplified can include mRNA or cDNA or genomic DNA prepared from any vertebrate species. One can choose to synthesize several different degenerate primers for  
30 use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence homology between the liver activin gene being cloned and the known liver activin gene. Other means  
35 for primer dependent amplification of nucleic acids are known to those of skill in the art and can be used.

The PCR product also may be subcloned, sequenced, and used to probe a Northern blot so as to ensure that the amplified sequences represent the sequences of a liver activin gene. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

10        PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source (*i.e.*, one known, or suspected, to express the liver activin gene, such as, for example, embryonic  
15 tissue, adult liver tissue, or ATCC human liver cell lines 7004, 7120, 7128, 7137, 7154, 7274, 7284, 7290, 7297, 7503, 7705, 7821, 7837, 7859 or 7864). A reverse transcription reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment  
20 for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanine using a standard terminal transferase reaction, the hybrid may be digested with RNAase H, and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences  
25 upstream of the amplified fragment may easily be isolated. For a review of cloning strategies which may be used, see *e.g.*, Sambrook et al., 1989, *supra*.

Alternatively, the liver activin gene of the present invention may be isolated through an exon trapping system,  
30 using genomic DNA (Nehls et al., 1994, *Oncogene* 9(8):2169-2175; Verna et al., 1993, *Nucleic Acids Res.* 21(22):5198:5202; Auch et al., 1990, *Nucleic Acids Res.* 18(22):6743-6744).

The liver activin gene sequences may additionally be  
35 used to isolate mutant liver activin gene alleles. Such mutant alleles may be isolated, for example, but not by way of limitation, from individuals either known or proposed to

have a genotype which contributes to cell growth and/or differentiation disorders. Mutant alleles and mutant allele products may then be utilized in the therapeutic and diagnostic systems described below. Additionally, such liver  
5 activin gene sequences can be used to detect liver activin gene regulatory (e.g., promoter or promoter/enhancer) defects which can affect cell growth and/or differentiation.

A cDNA of a mutant liver activin gene may be isolated, for example, by using PCR, a technique which is well known to  
10 those of skill in the art. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from liver tissue in an individual putatively carrying the mutant liver activin allele, and by extending the new strand with reverse  
15 transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence  
20 analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant liver activin allele to that of the normal liver activin allele, the mutation(s) responsible for the loss or alteration of function of the mutant liver activin gene product can be  
25 ascertained. Alternative hybridization or amplification assays for detecting abnormalities in the amplified liver activin gene fragment, include, but are not limited to, Southern analysis, single stranded conformational polymorphism analysis (SSCP), denaturing gradient gel  
30 analysis, and PCR analysis.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry the mutant liver activin allele, or a cDNA library can be constructed using RNA from liver or other tissue  
35 known, or suspected, to express the mutant liver activin allele. The normal liver activin gene or any suitable fragment thereof may then be labeled and used as a probe to

identify the corresponding mutant liver activin allele in such libraries. Clones containing the mutant liver activin gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from embryonic tissue or adult liver tissue known, or suspected, to express a mutant liver activin allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against the normal liver activin gene product, as described, below, in Section 5.3. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.) In cases where a liver activin mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), a polyclonal set of antibodies to liver activin are likely to cross-react with the mutant liver activin gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

The invention also encompasses nucleotide sequences that encode mutant liver activin proteins, polypeptides, derivatives (including peptide fragments) of the liver activin, truncated liver activins, and liver activin fusion proteins. These include, but are not limited to, truncated nucleotide sequences in which all or part of the nucleotides corresponding to the 3' untranslated region are deleted; nucleotide sequences encoding the mutant liver activins described in section 5.2 *infra*; polypeptides or peptides corresponding to the N-terminal signal peptide domain, propeptide domain, and mature growth factor domain of the liver activin or portions of these domains; and truncated

liver activins in which one or two of the domains is deleted, e.g., a mature growth factor domain lacking the N-terminal signal peptide and/or propeptide domain, or a truncated, nonfunctional liver activin lacking all or a portion of the  
5 endoproteolytic cleavage motif and/or N-terminal signal peptide domain, propeptide domain, and/or mature growth factor domain. Nucleotides encoding fusion proteins may include, but are not limited to, full length liver activin, truncated liver activin, polypeptides or derivatives  
10 (including fragments) of liver activin fused to an unrelated protein or peptide, such as, for example, a mature growth factor domain sequence; an Ig Fc domain which increases the stability and half life of the resulting fusion protein (e.g., liver activin-Ig) in the bloodstream; or an enzyme,  
15 fluorescent protein, or luminescent protein which can be used as a marker.

The invention also encompasses (a) DNA vectors that contain any of the foregoing liver activin coding sequences and/or their complements (i.e., antisense); (b) DNA  
20 expression vectors that contain any of the foregoing liver activin coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the foregoing liver activin coding sequences  
25 operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the  
30 art that drive and regulate expression. Such regulatory elements include, but are not limited to, the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter  
35 regions of phage A, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of

acid phosphatase, and the promoters of the yeast  $\alpha$ -mating factors.

In addition, the invention encompasses genomic nucleotide sequences found adjacent to the liver activin genes. Such genomic nucleotide sequences include those containing regulatory elements that act to drive and regulate expression of liver activins. The regulatory elements may be identified using routine techniques well known to those skilled in the art. For example, the genomic nucleotide sequences located upstream of the liver activin genes can be cloned adjacent to a reporter gene such as a CAT gene. The transcriptional activity of the nucleotide sequences may be determined by assaying for reporter gene activity. To more precisely define the regulatory elements, deletion mutants can be generated and tested for reporter gene activity.

#### 5.1.1 THE ACTIVIN $\beta_c$ GENE

The cDNA sequence (SEQ. ID. No. 1) and deduced amino acid sequence (SEQ. ID. No. 2) of murine activin  $\beta_c$  are shown in FIG. 1. The murine activin  $\beta_c$  signal sequence extends from amino acid residue 1 to about 20 of FIG. 1. A cluster of basic residues (KHRVRRR, amino acid 230-236) may serve as an endoproteolytic cleavage site that defines the predicted propeptide and mature growth factor domains. The propeptide domain consists of approximately 216 residues and extends from about amino acid residue 21 to about 236 of FIG. 1; and the mature growth factor domain consists of approximately 116 residues and extends from about amino acid residue 237 to about 352 of FIG. 1. Sequences derived from the murine activin  $\beta_c$  cDNA clone were used to screen mouse genomic DNA and liver cDNA libraries under low stringency conditions and to clone the gene corresponding to  $\beta_E$ , as described in the examples, infra.

### 5.1.2 THE ACTIVIN $\beta_E$ GENE

The cDNA sequence (SEQ. ID. No. 3) and deduced amino acid sequence (SEQ. ID. No. 4) of murine activin  $\beta_E$  are shown in FIG. 2. The murine activin  $\beta_E$  signal sequence extends from 5 amino acid residue 1 to about 21 of FIG. 2. A cluster of basic residues (RARRR, amino acid residues 232-236) may serve as an endoproteolytic cleavage site (or sites) that defines the predicted propeptide and mature growth factor domains. The propeptide domain consists of approximately 215 residues and extends from about amino acid residue 22 to about 236 of FIG. 2; and the mature growth factor domain of activin  $\beta_E$  subunit consists of approximately 113 residues and extends from about amino acid residue 237 to about 349 of FIG. 2.

### 15 5.1.3 THE HUMAN ACTIVIN $\beta_E$ GENE

The murine activin  $\beta_E$  cDNA clone was used to screen a human liver cDNA library under low stringency conditions and the cDNA sequence (SEQ. ID. NO. 5) and deduced amino acid sequence (SEQ. ID. NO. 6) of an isolated human activin  $\beta_E$  are 20 shown in FIG. 5. Sequence analysis revealed a predicted open reading frame of 1050 nucleotides coding for a protein with an estimated molecular weight of 39 kD. The putative methionine start codon is located at nucleotide 77. A canonical CXCX sequence, preceding the stop translation 25 signal (TAG) was also identified.

The human activin  $\beta_E$  subunit is organized like other TGF- $\beta$  superfamily members. The predicted initiation Met residue is followed by a characteristic signal peptide of ~20 amino acids. Downstream of the signal peptide, predicted 30 propeptide and mature growth factor domains were identified. The domains were separated by a cluster of basic amino acids, which create one or more putative endoproteolytic cleavage sites. If these cleavage site(s) are in fact used, then the propeptide and mature growth factor domains of the human 35 activin  $\beta_E$  subunit will consist of ~215 and 114 amino acids, respectively.

The predicted carboxy-terminal amino acid sequences of mouse and human activin  $\beta_E$  are highly homologous, with >95% amino acid identity over the mature growth factor region. Both coding sequences contain a total of nine cysteine  
5 residues. We found that the spacing of the cysteines and the presence of other conserved amino acids is most characteristic of the activins and, to a lesser extent, TGF- $\beta$  itself. In tabulating percenacid sequence identity within the mature growth factor region, human activin  $\beta_E$  showed 45%  
10 identity with human activin  $\beta_A$ , 47% identity with human activin  $\beta_B$ , 64% identity with human activin  $\beta_C$ , and 62% identity with *Xenopus* activin  $\beta_D$ . In contrast, only 20-40% amino acid sequence identity was found when human activin  $\beta_E$  was compared with other members of the TGF- $\beta$  superfamily. To  
15 our knowledge, this is the first report of the isolation of activin  $\beta_E$  from human.

#### 5.1.4 THE MOUSE ACTIVIN GENETIC LOCUS

A genetic map of the mouse activin genetic locus is  
20 shown in FIG. 4A. The nucleotide sequence of the murine activin genetic locus derived from a genomic mouse clone is shown in FIG. 4C. Our studies suggest that in certain respects activin  $\beta_C$  and  $\beta_E$  are more like one another than other known activins: (a), these genes are physically  
25 linked, with only 5-kbp of genomic DNA separating the 3' end of activin  $\beta_C$  from the 5' end of activin  $\beta_E$ ; (b), the organization of both genetic loci is similar, consisting of two coding exons separated by a single intervening sequence; (c), the predicted carboxy-terminal sequences of both  
30 activins are highly homologous, with 62% amino acid identity over the mature growth factor region; (d), both genes appear to be specifically and highly expressed in adult mouse liver and (e) the results of several in vitro assays show that homodimers of activin  $\beta_C$  and  $\beta_E$  stimulate the proliferation of  
35 HepG2 cells. When taken together, our data suggest that the activin  $\beta_C$  and  $\beta_E$  subunit genes may have evolved from a common ancestor that underwent a tandem duplication. These

structural and functional data differentiate activin  $\beta_c$  and  $\beta_e$  subunits from other known activins, suggesting that these subunits represent a unique subgroup of TGF- $\beta$ -like molecules.

## 5                    5.2   LIVER ACTIVIN PROTEINS AND POLYPEPTIDES

10                    Liver activin protein, polypeptides and derivatives (including fragments); mutated, truncated or deleted forms of the liver activin; and/or liver activin fusion proteins can be prepared for a variety of uses. By way of example but not  
10 limitation, these include the generation of antibodies, which can be used in diagnostic assays; and as pharmaceutical reagents useful in the regulation of cell growth and/or differentiation and/or treatment of disorders related thereto.

15                    The sequence of liver activins  $\beta_c$  and  $\beta_e$  begins with a methionine in a DNA sequence context consistent with a translation initiation site, followed by a typical hydrophobic signal sequence of peptide secretion. The predicted propeptide and mature growth factor domains of  
20 mouse activin  $\beta_c$  and  $\beta_e$  are approximately 216 and 115 amino acids and 215 and 113 amino acids, respectively. The predicted propeptide and mature growth factor domains of human activin  $\beta_e$  are approximately 215 and 114 amino acids. Like all known TGF- $\beta$  superfamily members, except for MIS,  
25 activin  $\beta_c$  and  $\beta_e$  have a cluster of basic residues approximately 120 amino acids from the C-terminus, which form an endoproteolytic cleavage site at the junction of the propeptide domain and mature growth factor domain. (Roberts B, Sporn MB 1900, The Transforming Growth Factor- $\beta$ s in  
30 Handbook of Experimental Pharmacology, 95 (Part I) Sporn MB, Roberts AB eds., Springer-Verlag, Heidelberg, pp. 419-472; Vale W, Hsueth A, River J, Yu J 1990 in Peptide Growth Factors and Their Receptors, 95 (Part II) Sporn MB, Roberts AB eds, Springer-Verlag, Heidelberg, pp. 211-248; and  
35 Centrella et al., 1994, Endocr Rev 15:27-38). An alignment between the mature growth factor domain of liver activin  $\beta_c$  and  $\beta_e$  and other activins and other TGF- $\beta$  superfamily members

is shown in FIG. 3. The liver activin mature growth factor domain contains nine cysteine residues which demonstrate a pattern of spacing that is conserved among TGF- $\beta$  superfamily members. While showing significant homology to activins  $\beta_A$  and  $\beta_B$ , liver activins  $\beta_C$  and  $\beta_E$  are most closely related to activin  $\beta_D$ .

FIG. 1 shows the amino acid sequence of the murine liver activin  $\beta_C$  subunit. The signal sequence extends from amino acid 1 to 20. A cluster of basic residues (KHRVRRR, amino acid 230-236) may serve as an endoproteolytic cleavage site(s) that defines the predicted propeptide and mature growth factor domains. The propeptide domain consists of approximately 216 residues and extends from about amino acid residue 21 to about 236. The mature growth factor domain consists of approximately 115 residues and extends from about amino acid residue 237 to about 352 of FIG. 1. FIG. 2 shows the amino acid sequence of the murine liver activin  $\beta_E$  protein. The signal sequence extends from amino acid residue 1 to 21. A cluster of basic residues (RARRR, amino acid residues 232-236) may serve as an endoproteolytic cleavage site (or sites) that defines the predicted propeptide and mature growth factor domains. The propeptide domain consists of approximately 215 residues and extends from about amino acid residue 22 to about 236. The mature growth factor domain consists of approximately 113 residues and extends from about amino acid residue 237 to about 349.

FIG. 5 shows the amino acid sequence of human liver activin  $\beta_E$  subunit. The human activin  $\beta_E$  subunit is organized like other TGF- $\beta$  super family members. The predicted initiator MET residue is followed by a characteristic signal peptide of ~ 20 amino acids. A cluster of basic amino acids were identified which create endoproteolytic cleavage site(s) that define the predicted propeptide and mature growth factor domains. Once cleaved, the propeptide and mature growth factor domains of human activin  $\beta_E$  will consist of approximately 215 and 114 amino acids, respectively.

Potential N-linked glycosylation sites (i.e., amino acid sequence motif N-X-S or N-X-T) are found in the propeptide domains of both murine  $\beta_c$  and  $\beta_e$  liver activin subunits and human  $\beta_e$  liver activin subunits. Four potential N-linked glycosylation sites can be identified in the murine  $\beta_c$  sequence shown in FIG. 1 (see tripeptide motifs starting at amino acid residues 111, 143, 161 and 173), whereas one potential N-linked glycosylation site can be identified in the murine  $\beta_e$  sequence shown in FIG. 2 (see the tripeptide motif starting at amino acid residue 198).

The liver activin amino acid sequences of the invention include the amino acid sequence shown in FIG. 1 (SEQ. ID. No. 2), FIG. 2 (SEQ. ID. No. 4), and FIG. 5 (SEQ. ID. NO.: 6) and the amino acid sequence encoded by cDNA clone pbC as deposited with the ATCC, cDNA clone pbE as deposited with the ATCC, and cDNA clone pbHE as deposited with the ATCC. Further, other murine and human liver activins and liver activin homologs are encompassed by the invention. In fact, any liver activin protein encoded by the liver activin nucleotide sequences described in Section 5.1, above, is within the scope of the invention. Such animals may be produced for use as "bioreactors" for production of liver activins.

The invention also encompasses proteins that are functionally equivalent to the liver activin encoded by the nucleotide sequences described in Section 5.1, as judged by any of a number of criteria, including but not limited to (a) antigenicity, i.e., the ability to bind to an anti-liver activin antibody; (b) immunogenicity, i.e., the ability to generate an antibody which is capable of binding a liver activin protein or polypeptide; (c) the ability to bind (or compete with liver activin for binding) to a substrate for liver activin; (d) ability to multimerize with liver activin; and (e) the ability to bind a liver activin receptor, the binding affinity for an activin receptor, the resulting biological effect of binding to an activin receptor, e.g., signal transduction, a change in cellular metabolism (e.g.,

ion flux, tyrosine phosphorylation) or change in phenotype when the liver activin equivalent is present in an appropriate cell type (such as the stimulation or inhibition of cellular growth and/or differentiation). Such

5 functionally equivalent liver activin proteins include but are not limited to additions or substitutions of amino acid residues within the amino acid sequence encoded by the liver activin nucleotide sequences described above, in Section 5.1, but which result in a silent change, thus producing a

10 functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine,

15 leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged

20 (acidic) amino acids include aspartic acid and glutamic acid.

Included within the scope of the invention are liver activin proteins, polypeptides, and derivatives (including fragments) which are differentially modified during or after translation, e.g., by glycosylation, acetylation,

25 phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical

30 cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease,  $\text{NaBH}_4$ ; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc. In a specific embodiment, the compositions of the invention are conjugated to other molecules to

35 increase their water-solubility (e.g., polyethylene glycol), half-life, or ability to bind targeted tissue (e.g.,

bisphosphonates and fluorochromes to target the proteins to bony sites).

Furthermore, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition  
5 into the liver activin sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids,  $\alpha$ -amino isobutyric acid; 4-aminobutyric acid, Abu; 2-amino butyric acid,  $\gamma$ -Abu;  $\epsilon$ -Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-amino propionic acid;  
10 ornithine; norleucine; norvaline; hydroxyproline; sarcosine; citrulline; cysteic acid; t-butylglycine; t-butylalanine; phenylglycine; cyclohexylalanine;  $\beta$ -alanine; fluoro-amino acids; "designer" amino acids such as  $\beta$ -methyl amino acids; C $\alpha$ -methyl amino acids; N $\alpha$ -methyl amino acids; and amino acid  
15 analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

While random mutations can be applied to liver activin DNA (using techniques well known to those skilled in the art) and the resulting mutant liver activins tested for activity,  
20 site-directed mutations of the liver activin coding sequence can be engineered (again using techniques well known to those skilled in the art) to generate mutant liver activins with higher or lower binding affinity for binding proteins, and/or increased function, e.g., higher binding affinity for liver  
25 activin receptors, and/or greater signalling capacity; or decreased function, e.g., lower binding affinity for liver activin receptors, and/or decreased signalling capacity.

For example, the alignment of liver activins and other activin group members is shown in FIG. 3. Mutant liver  
30 activins can be engineered so that regions of identity are maintained, whereas the variable residues are altered, e.g., by deletion or insertion of an amino acid residue(s) or by substitution of one or more different amino acid residues. Conservative alterations at the variable positions can also  
35 be engineered in order to produce a mutant liver activin that retains function, e.g., liver activin receptor binding affinities or signalling capability or both. Non-

conservative changes can be engineered at these variable positions to alter function, e.g., liver activin binding affinities or signalling capability, or both. Alternatively, where alteration of function is desired, deletion or non-  
5 conservative alterations of the conserved regions can be engineered. For example, deletion or non-conservative alterations (substitutions or insertions) of amino acid residues 21-236 (FIG. 1) of murine liver activin  $\beta_c$ , amino acid residues 22-236 (FIG. 2) of murine liver activin  $\beta_e$ ,  
10 amino acid residues 17 to about 236 (FIG. 5) of human liver activin  $\beta_e$  or amino acid residues 237-352 (FIG. 1) of murine liver activin  $\beta_c$ , amino acid residues 237-349 (FIG. 2) of murine activin  $\beta_e$  or amino acid residues 237-350 (FIG. 5) of human liver activin  $\beta_e$  can be engineered to produce a mutant  
15 liver activin that is unable to form dimers with  $\alpha$  and/or  $\beta$  subunits, and thus is unable to achieve a functional structural conformation, or that binds liver activin receptor but is signalling-incompetent. Non-conservative alterations to the cysteine residues in the mature growth factor domain  
20 shown in FIG. 3 can be engineered to produce mutant liver activins with altered structure and thus altered binding affinity for liver activin receptor.

Other mutations to the liver activin coding sequence can be made to generate liver activins that are better suited for  
25 expression, scale up, etc. in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid in order to eliminate disulfide bridges; or N-linked glycosylation sites can be altered or eliminated to achieve expression of a homogeneous product that is more  
30 easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety of amino acid substitutions at one or both of the first or third amino acid positions of any one or more of the glycosylation recognition sequences which occur in the liver  
35 activin propeptide domain (N-X-S or N-X-T) will prevent glycosylation of the propeptide at the modified tripeptide

sequence. See, e.g., Miyajima et al., 1986, EMBO J. 5(6):1193-1197.

Peptides corresponding to one or more domains of liver activin (e.g., N-terminal signal peptide, propeptide, or  
5 mature growth factor); truncated or deleted liver activins (e.g., molecules in which signal and/or propeptide domain is deleted); and fusion proteins in which the full length liver activin protein, polypeptide or derivative (including fragment), or truncated liver activin is fused to an  
10 unrelated protein are also within the scope of the invention. Altered activins can be designed on the basis of the liver activin nucleotide and liver activin amino acid sequences disclosed in this Section and in Section 5.1, above. The fusion protein may also be engineered to contain a cleavage  
15 site located between a liver activin sequence and the non-liver activin protein sequence, so that the liver activin protein may be more conveniently cleaved away from the non-liver activin moiety. Such fusion proteins include, but are not limited to, IgFc fusions, which stabilize the liver  
20 activin protein or peptide and prolong half-life in vivo; or fusions to an enzyme, fluorescent protein, or luminescent protein which provide a marker function.

While the liver activin proteins, polypeptides and derivatives (including fragments) can be chemically  
25 synthesized (e.g., see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y.), large polypeptides derived from the liver activin, and the full length liver activin itself, may advantageously be produced by recombinant DNA technology using techniques well known in  
30 the art for expressing gene sequences and/or nucleic acid coding sequences. Such methods can be used to construct expression vectors containing the liver activin nucleotide sequences described in Section 5.1 and appropriate transcriptional and translational control signals. These  
35 methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. See, for example, the techniques described in

Sambrook et al., 1989, supra; Ausubel et al., 1989, supra;  
Caruthers et al., 1980, Nuc. Acids Res. Symp. Ser. 7:215-233;  
Crea and Horn, 1980, Nuc. Acids Res. 9(10):2331; Matteucci  
and Caruthers, 1980, Tetrahedron Letters 21:719; and Chow and  
5 Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively,  
RNA capable of encoding liver activin nucleotide sequences  
may be chemically synthesized using, for example,  
synthesizers. See, for example, the techniques described in  
"Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press,  
10 Oxford, which is incorporated by reference herein in its  
entirety.

A variety of host-expression vector systems may be  
utilized to express the liver activin nucleotide sequences of  
the invention. The liver activin protein, polypeptide or  
15 derivative can be recovered from the culture, i.e., from the  
host cell in cases where the liver activin peptide or  
polypeptide is not secreted, and from the culture media in  
cases where the liver activin protein, polypeptide or  
derivative is secreted by the cells. However, the expression  
20 systems also encompass engineered host cells that express the  
liver activin or functional equivalents in situ.

The expression systems that may be used for purposes of  
the invention include, but are not limited to, bacterial cell  
systems (e.g., E. coli, B. subtilis) transformed with  
25 recombinant bacteriophage DNA, plasmid DNA or cosmid DNA  
expression vectors containing liver activin nucleotide  
sequences; yeast cell systems (e.g., Saccharomyces, Pichia)  
transformed with recombinant yeast expression vectors  
containing the liver activin nucleotide sequences; insect  
30 cell systems infected with recombinant virus expression  
vectors (e.g., baculovirus) containing the liver activin  
sequences; plant cell systems infected with recombinant virus  
expression vectors (e.g., cauliflower mosaic virus, CaMV;  
tobacco mosaic virus, TMV) or transformed with recombinant  
35 plasmid expression vectors (e.g., Ti plasmid) containing  
liver activin nucleotide sequences; or mammalian cell systems  
(e.g., COS, CHO, BHK, 293, 293T, 3T3) harboring recombinant

expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

5 In bacterial systems, a number of expression vectors may be advantageously chosen depending upon the use intended for the liver activin gene product being expressed. When a large quantity of such a protein is to be produced, as for example during drug manufacturing or to raise antibodies, vectors  
10 which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the liver activin coding sequence may be  
15 ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); the pcDNA (Invitrogen) eukaryotic vectors, and the  
20 like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution  
25 in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear  
30 polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The liver activin gene coding sequence may be cloned individually into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an  
35 AcNPV promoter (for example, the polyhedrin promoter). Successful insertion of liver activin gene coding sequence will result in inactivation of the polyhedrin gene and

production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is  
5 expressed. (E.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the liver activin  
10 nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-  
15 essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the liver activin gene product in infected hosts. E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659. Specific initiation signals may also be  
20 required for efficient translation of inserted liver activin nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire liver activin gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the  
25 appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the liver activin coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the  
30 initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be  
35 enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. See Bittner et al., 1987, Methods in Enzymol. 153:516-544.

In addition, a host cell strain may be chosen which modulates the expression of inserted sequences, or which modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 293T, 3T3, WI38, and in particular, cell lines generated from embryonic tissue or adult liver.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the liver activin sequences described above may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by a selectable marker and appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci, which, in turn, can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which overexpress the liver activin gene product in vitro.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc.

Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk<sup>-</sup>, hgp<sup>r</sup>t<sup>-</sup> or ap<sup>r</sup>t<sup>-</sup> cells, respectively. Also, antimetabolite resistance can be used as  
5 the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl.  
10 Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg<sup>r</sup>, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147).

Alternatively, any fusion protein may be readily  
15 purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-  
20 8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are  
25 loaded onto Ni<sup>2+</sup>-nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

The liver activin gene products can also be expressed in transgenic animals. Animals of any species, including, but  
30 not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate liver activin transgenic animals.

Any technique known in the art may be used to introduce  
35 the liver activin transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection

(Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells  
5 (Thompson et al., 1989, Cell 56:313-321); and electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814). For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

10       The present invention provides for transgenic animals that carry the liver activin transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals. The transgene may be integrated as a single transgene or in concatamers, e.g.,  
15 head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad. Sci. USA 89:6232-6236). The regulatory sequences required  
20 for such a cell-type specific activation will depend upon the particular cell type of interest and will be apparent to those of skill in the art. When it is desired that the liver gene transgene be integrated into the chromosomal site of the endogenous liver activin gene, gene targeting is preferred.  
25 Briefly, when such a technique is to be utilized, vectors containing nucleotide sequences homologous to the endogenous liver activin gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, and disrupting the function of the endogenous  
30 liver activin gene product. The transgene may also be selectively introduced into a particular cell type such as liver, thus inactivating the endogenous liver activin gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu, et al., 1994, Science 265:103-106). The  
35 regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant liver activin gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques  
5 to analyze animal tissues for integration of the transgene. The tissue level of transgene mRNA expression may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and RT-PCR.  
10 Samples of liver activin gene-expressing tissue may also be evaluated immunocytochemically using antibodies specific for the liver activin transgene product.

### 5.3 ANTIBODIES TO LIVER ACTIVIN PROTEINS

15 Antibodies that specifically recognize one or more epitopes of liver activin, or epitopes of conserved variants of liver activin, or peptide fragments of the liver activin, are also encompassed by the invention. Such antibodies include, but are not limited to, polyclonal antibodies,  
20 monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.  
25 The antibodies of the invention may be used, for example, in the detection of the liver activin in a biological sample and, therefore, may represent part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of liver activin. Such  
30 antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described below in Section 5.5, for the evaluation of the effect of test compounds on expression and/or activity of the liver activin gene product. Additionally, such antibodies can be used in  
35 conjunction with the gene therapy techniques, described below in Section 5.6, to, for example, evaluate the normal and/or engineered liver activin-expressing cells prior to their

introduction into the patient. Such antibodies may additionally be used as part of a method to regulate and/or inhibit liver activin activity. Such antibodies, therefore, may be utilized as part of a treatment method to achieve cell  
5 growth and/or differentiation.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals. For the production of antibodies, various host animals may be immunized by injection with the liver activin  
10 protein; a liver activin polypeptide or liver activin derivative (e.g., one corresponding to a functional domain of the liver activin, such as the mature growth factor domain); a truncated liver activin polypeptide (liver activin in which one or more domains, e.g., the N-terminal signal peptide or  
15 propeptide domain has been deleted); a functional equivalent of liver activin; or mutants of a liver activin. Such host animals may include, but are not limited to, rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host  
20 species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human  
25 adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody  
30 molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al.,  
35 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such

antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454), by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity, can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce single chain antibodies against liver activin gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab')_2$  fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the  $F(ab')_2$  fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to the liver activin can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic"

the liver activin or to identify the liver activin cell surface receptor, using techniques well known to those skilled in the art. See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438. For example, antibodies which bind to the liver activin mature growth factor domain and competitively inhibit the binding of the liver activin receptor to the liver activin can be used to generate anti-idiotypes that "mimic" the mature growth factor region and, therefore, bind and neutralize liver activin receptor. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens that either interfere with, stimulate, or neutralize the effect of liver activin on cell growth and/or differentiation.

15

#### 5.4 DIAGNOSIS OF CELL GROWTH AND/OR DIFFERENTIATION ABNORMALITIES

A variety of methods can be employed for the diagnostic and prognostic evaluation of cell growth and/or differentiation disorders, and for the identification of subjects having a predisposition to such disorders. Such disorders include, but are not limited to, disorders in hematopoiesis, erythroid differentiation, ovarian follicular maturation, pituitary growth hormone and adrenocortical tropic hormone secretion, hypothalamic oxytocin secretion, lymphocyte proliferation, neuronal survival, spermatogenesis, bone and growth formation, hepatocyte DNA synthesis, pancreatic insulin secretion, somatostatin induction, cardiac morphogenesis, megakaryocytic differentiation and the induction of mesoderm during early embryonic development. Cell growth and/or differentiation disorders which may be diagnosed and prognosed according to the methods of the present invention include but are not limited to disorders in hematopoiesis, erythropoiesis, ovarian follicular maturation, pituitary growth hormone and adrenocortical tropic hormone secretion, hypothalamic oxytocin secretion, lymphocyte proliferation, neuronal survival, spermatogenesis, bone

formation, hepatocyte DNA synthesis, pancreatic insulin secretion, and the induction of mesoderm during early embryonic development.

Such methods may, for example, utilize reagents such as  
5 the liver activin nucleotide sequences described in Section 5.1, and liver activin antibodies, as described, in Section 5.3. Specifically, such reagents may be used to, for example: (1) detect the presence of liver activin gene mutations, or detect either over- or under-expression of  
10 liver activin mRNA relative to the normal cell growth and/or differentiation state; (2) detect either an over- or an under-abundance of liver activin gene product relative to the non-cell growth and/or non-differentiation disorder state; and (3) detect perturbations or abnormalities in the signal  
15 transduction pathway mediated by liver activin ligand.

The methods described herein may be performed, for example, with pre-packaged diagnostic kits comprising at least one specific liver activin nucleotide sequence or liver activin antibody reagent described herein. Such kits may be  
20 conveniently used, e.g., in clinical settings, to diagnose patients exhibiting cell growth and/or differentiation disorder abnormalities.

For the detection of liver activin gene mutations, any nucleated cell can be used as a starting source for genomic  
25 DNA. To detect liver activin gene expression or liver activin gene products, any cell type or tissue in which the liver activin gene is expressed, such as, for example, adult liver, may be utilized.

Nucleic acid-based detection techniques are described,  
30 below, in Section 5.4.1. Peptide detection techniques are described, below, in Section 5.4.2.

#### 5.4.1 DETECTION OF THE LIVER ACTIVIN/ INHIBIN GENE AND TRANSCRIPTS

Mutations within the liver activin gene can be detected by utilizing a number of techniques. DNA from any nucleated cell can be used as the starting point for such assay techniques. DNA may be isolated according to standard nucleic acid preparation procedures, which are well known to those of skill in the art.

DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving liver activin gene structure, including point mutations, insertions, deletions, trinucleotide repeat expansions and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analysis, single stranded conformational polymorphism analysis (SSCP), denaturing gradient gel analysis, and PCR analysis.

Diagnostic methods to detect liver activin gene-specific mutations can involve, for example, incubating nucleic acids (including recombinant DNA molecules, cloned genes or degenerate variants thereof), obtained from a sample, *e.g.*, derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid probes including recombinant DNA molecules, cloned genes or degenerate variants thereof, as described in Section 5.1, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the liver activin gene. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid:liver activin molecule hybrid. The presence of nucleic acids which have hybridized, if any such molecules exist, is then detected. For one such detection scheme, the nucleic acid from the cell type or tissue of interest is immobilized, for example, to a solid support such as a membrane, or a plastic surface such as a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents of the type described

in Section 5.1 are easily removed with simple washing procedures. Detection of the annealed, labeled liver activin nucleic acid reagents is accomplished using standard techniques well-known to those in the art. The structure  
5 and/or level of expression of liver activin gene sequences to which the nucleic acid reagents have annealed can be compared to the annealing pattern expected from normal in order to determine whether a liver activin gene mutation is present.

Alternative diagnostic methods to detect liver activin  
10 gene specific nucleic acid molecules, in patient samples or other appropriate cell sources, may involve their amplification, e.g., by PCR (the experimental embodiment set forth in Mullis, K.B., 1987, U.S. Patent No. 4,683,202), followed by the evaluation of the amplified molecules using  
15 techniques well known to those of skill in the art. The resulting amplified sequences can be compared, for example, to normal in order to determine whether a liver activin gene mutation exists.

Additionally, well-known genotyping techniques can be  
20 performed to identify individuals carrying liver activin gene mutations. Such techniques include, for example, the use of restriction fragment length polymorphisms (RFLPs), which involve sequence variations in one of the recognition sites for the specific restriction enzyme used.

25 Additionally, improved methods for analyzing DNA polymorphisms, which can be utilized for the identification of liver activin gene mutations, have been described which capitalize on the presence of variable numbers of short, tandemly repeated DNA sequences between the restriction  
30 enzyme sites. For example, Weber (U.S. Pat. No. 5,075,217, which is incorporated herein by reference in its entirety) describes a DNA marker based on length polymorphisms in blocks of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> short tandem repeats. The average separation of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> blocks is estimated  
35 to be 30,000-60,000 bp. Markers which are so closely spaced exhibit a high frequency co-inheritance, and are extremely useful in the identification of genetic mutations, such as,

for example, mutations within the liver activin gene, and the diagnosis of diseases and disorders related to liver activin mutations.

Also, Caskey et al. (U.S. Pat. No. 5,364,759, which is  
5 incorporated herein by reference in its entirety) describe a  
DNA profiling assay for detecting short tri and tetra  
nucleotide repeat sequences. The process includes extracting  
the DNA of interest, such as the liver activin gene,  
amplifying the extracted DNA, and labeling the repeat  
10 sequences to form a genotypic map of the individual's DNA.

The level of liver activin gene expression can also be  
assayed by detecting and measuring liver activin  
transcription. For example, RNA from a cell type or tissue  
known, or suspected, to express the liver activin gene, such  
15 as adult liver, may be isolated and tested with hybridization  
or PCR techniques such as those described above. The  
isolated cells can be derived from cell culture or directly  
from a patient. The analysis of cells taken from culture may  
be a necessary step in the assessment of cells to be used as  
20 part of a cell-based gene therapy technique or,  
alternatively, to test the effect of compounds on the  
expression of the liver activin gene. Such analyses may  
reveal both quantitative and qualitative aspects of the  
expression pattern of the liver activin gene, including  
25 activation or inactivation of the liver activin gene  
expression.

In one embodiment of such a detection scheme, cDNAs are  
synthesized from the RNAs of interest (e.g., by reverse  
transcription of the RNA molecule into cDNA). A sequence  
30 within the cDNA is then used as the template for a nucleic  
acid amplification reaction, such as a PCR amplification  
reaction, or the like. Oligonucleotide primers for reverse  
transcription and nucleic acid amplification are chosen from  
among the liver activin nucleic acid reagents described in  
35 Section 5.1. The preferred lengths of such nucleic acid  
reagents are at least 9-30 nucleotides. To detect the  
amplified product, the nucleic acid amplification may be

performed using radioactively or non-radioactively labeled nucleotides. Alternatively, Southern analysis may be performed, or enough amplified product may be generated such that the product may be visualized by standard ethidium  
5 bromide staining or by utilizing any other suitable nucleic acid staining method.

Additionally, it is possible to perform such liver activin gene expression assays "in situ", i.e., directly upon histological sections (fixed and/or frozen) of patient tissue  
10 obtained from biopsies or resections, such that no nucleic acid purification is necessary. Oligonucleotides such as those described in Section 5.1 may be used as probes and/or primers for such in situ procedures (See, for example, Nuovo, G.J., 1992, "PCR In Situ Hybridization: Protocols And  
15 Applications", Raven Press, NY).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained, Northern analysis can be performed to determine the level of mRNA expression of the liver activin gene.

20

#### 5.4.2 DETECTION OF THE LIVER ACTIVIN GENE PRODUCTS

Antibodies directed against wild type or mutant liver activin gene products or conserved variants or peptide  
25 fragments thereof, as discussed above in Section 5.3, may also be used as diagnostics and prognostics for cell growth and/or differentiation disorders as described herein. Such diagnostic methods may be used to detect abnormalities in the level of liver activin gene expression, or abnormalities in  
30 the structure and/or temporal, tissue, cellular, or subcellular location of the liver activin; and may be performed in vivo or in vitro, such as, for example, on serum or biopsy tissue.

For example, antibodies directed to epitopes of the liver activin mature growth factor domain can be used in vivo  
35 to detect the distribution and level of the liver activin protein in the body. Such antibodies can be labeled, e.g.,

with a radio-opaque or other appropriate compound, and then injected into a subject, in order to visualize binding to the liver activin expressed in the body, using methods such as X-rays, CAT-scans, or MRI. Labeled antibody fragments, e.g.,  
5 the Fab or single chain antibody comprising the smallest portion of the antigen binding region, are preferred for this purpose to promote crossing the blood-brain barrier and to permit labeling of liver activins in brain tissue.

Alternatively, immunoassays can be utilized on serum  
10 and/or autopsy samples in vitro to assess liver activin expression. Such biopsy and autopsy assays can utilize antibodies directed to various liver activin epitopes, e.g., epitopes within the N-terminal signal peptide, propeptide and/or mature growth factor domains. The use of labeled  
15 antibodies will yield useful information regarding translation, intracellular transport, and secretion of the liver activin, and can identify defects in processing.

The tissues or cell types to be analyzed will generally include those which are known, or suspected, to express the  
20 liver activin gene, such as, for example, the adult liver. The protein isolation methods employed herein may, for example, be those described in Harlow and Lane (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New  
25 York), which is incorporated herein by reference in its entirety. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step toward the development of a cell-based gene therapy protocol or, alternatively, to test  
30 the effect of compounds on liver activin gene expression and/or activity.

For example, antibodies, or fragments of antibodies, such as those described above in Section 5.3, may be used to quantitatively or qualitatively detect the presence of liver  
35 activin gene products or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently

labeled antibody (see below, this Section) coupled with light microscopic or fluorimetric detection.

The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed  
5 histologically, as in immunofluorescence, immunoelectron microscopy or non-immuno assays, for in situ detection of liver activin gene products or conserved variants or peptide fragments thereof.

In situ detection may be accomplished by screening blood  
10 serum or alternatively by removing a histological specimen from a patient, and applying thereto a labeled antibody of the present invention. For histological examination, the antibody (or fragment) is preferably applied by standard immunohistochemical methods. By this procedure, it is  
15 possible to determine not only the presence of the liver activin gene product, or conserved variants or peptide fragments, or the binding of liver activin to a receptor, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily  
20 perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

Immunoassays for liver activin gene products, or conserved variants or peptide fragments thereof, will  
25 typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying liver activin gene products, or conserved  
30 variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as  
35 nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by

treatment with the labeled liver activin antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support may then be detected by conventional means.

5 By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier is that it can be either soluble (to some extent) or insoluble in aqueous environments for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of liver activin antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the liver activin antibody can be detectably labeled is by linking the same to an enzyme for subsequent use in an enzyme immunoassay (EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, MD); Voller, A. et al., 1978, J. Clin. Pathol. 31:507-520; Butler, J.E., 1981, Meth. Enzymol. 73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, FL; Ishikawa, E. et al.,

(eds.), 1981, Enzyme Immunoassay, Kigaku Shoin, Tokyo). The enzyme that is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be  
5 detected, for example, by spectrophotometric, fluorimetric or visual means. Enzymes that can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-  
10 glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by  
15 colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a  
20 variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect liver activin through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand  
25 Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter, scintillation counter or phosphorimager, or by autoradiography.

30 It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are  
35 fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthalaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as  $^{152}\text{Eu}$ , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as  
5 diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by  
10 detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

15 Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems, in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent  
20 protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

#### 25 5.5 SCREENING ASSAYS FOR COMPOUNDS THAT MODULATE LIVER ACTIVIN EXPRESSION OR ACTIVITY

The following assays are designed to identify compounds that interact with (e.g., bind to) liver activin (including, but not limited to, the mature growth factor domain of liver  
30 activin), compounds that interfere with or enhance the interaction of liver activin with its cognate signaling receptors, and to compounds which modulate the activity of the liver activin gene (i.e., modulate the level of liver  
35 activin gene expression) or modulate the level of liver activin activity. Assays may also be used that identify compounds which bind to liver activin gene regulatory sequences (e.g., promoter sequences) and which may modulate

liver activin gene expression. See e.g., Platt, K.A., 1994, J. Biol. Chem. 269:28558-28562, which is incorporated herein by reference in its entirety.

The compounds that may be screened in accordance with  
5 the invention include, but are not limited to, peptides, antibodies and fragments thereof, and other organic compounds (e.g., peptidomimetics) that bind to the propeptide or mature growth factor domain of liver activin. Peptides, antibodies or fragments thereof, and other organic compounds that mimic  
10 the mature growth factor domain of the liver activin (or a portion thereof), which bind to and "neutralize" natural liver activin receptor and thereby either mimic the activity triggered by the liver activin (i.e., agonists) or inhibit the activity triggered by the liver activin (i.e.,  
15 antagonists) may also be screened in accordance with the invention.

Such compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to those found in random peptide libraries;  
20 (see, e.g., Lam, K.S. et al., 1991, Nature 354:82-84; Houghten, R. et al., 1991, Nature 354:84-86). Such compounds may also be found in combinatorial chemistry-derived molecular libraries made of D- and/or L- configuration amino acids; phosphopeptides (including, but not limited to members  
25 of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang, Z. et al., 1993, Cell 72:767-778); antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')<sub>2</sub> and FAb expression library  
30 fragments, and epitope-binding fragments thereof); and small organic or inorganic molecules.

Numerous experimental methods may be used to select and detect proteins that bind with liver activins and thereby modulate liver activin expression or activity, including, but  
35 not limited to, protein affinity chromatography, affinity blotting, immunoprecipitation, cross-linking, and library based methods such as protein probing, phage display and the

two-hybrid system. See generally, Phizicky, E., et al., 1995, Microbiol. Rev. 59:94-123. For example, the two-hybrid system may be used to detect interaction between liver activins and candidate proteins for which genes encoding the

5 candidate proteins are available by constructing the appropriate hybrids and testing for reporter gene activity. If an interaction is detected using the two-hybrid method, deletions can be made in the DNA encoding the candidate interacting protein or the liver activin protein to identify

10 a minimal domain for interaction. Alternatively, the two-hybrid system can be used to screen available organismal and/or mammalian tissue specific libraries of activation domain hybrids to identify proteins that bind to a liver activin. These screens result in the immediate availability

15 of the cloned gene for any new protein identified. In addition, since multiple clones that encode overlapping regions of protein are often identified, the minimal domain for interaction may be readily apparent from the initial screen.

20 Computer modelling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can modulate liver activin expression or activity. Having identified such a compound or composition, the active sites or regions are identified.

25 Such active sites might typically be ligand or receptor binding sites, such as the interaction domains of liver activin with its signaling receptor. The active site can be identified using methods known in the art including, for example, a comparison of the conceptual amino acid or

30 nucleotide sequences. It can also be identified by study of complexes of the relevant ligand and receptor. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site.

Next, the three dimensional geometric structure of the

35 active site is determined. This can be done by known methods, including X-ray crystallography, which can determine a complete molecular structure at high resolution. On the

other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Other experimental methods can be used to obtain partial or complete geometric structures. The geometric structures may also be obtained with a complexed ligand or receptor, natural or artificial, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modelling can be used to complete the structure or improve its accuracy. Any recognized modelling method may be used, including parameterized models specific to particular biopolymers, such as proteins or nucleic acids. These models include molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Finally, having determined the structure of the active site, either by experimentation, by modeling, or by a combination of the two, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer assisted. These compounds found from this search are potential liver activin modulating compounds.

Alternatively, these methods can be used to identify improved modulating compounds from an already known modulating compound or ligand or receptor, such as, for example, follistatin, latent TGF- $\beta$  binding proteins ("LTBP's"), or activin receptor. The composition of the

known compound, ligand, or receptor can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied to the new composition. The altered structure  
5 is then compared to the active site structure of the compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating compounds, ligands, or receptors of  
10 improved specificity or activity.

Further experimental and computer modeling methods useful to identify modulating compounds based upon identification of the active sites of liver activins, accessory proteins, liver activin receptors, and related  
15 transduction and transcription factors will be apparent to those of skill in the art.

Examples of molecular modeling systems are the CHARMM and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMM performs the energy minimization and molecular  
20 dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs  
25 interactive with specific proteins, such as Rotivinen, et al., 1988, Acta Pharmaceutical Fennica 97:159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinaly and Rossmann, 1989, Annu. Rev. Pharmacol. Toxicol. 29:111-122; Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug  
30 Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 Proc. R. Soc. Lond. 236:125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., 1989, J. Am. Chem. Soc. 111:1082-1090. Other computer programs that screen and graphically depict  
35 chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario). Although

these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

5        Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are liver  
10 activin inhibitors or activators.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the liver activin gene product, and for regulating cell growth and/or differentiation.

15

#### 5.5.1 IN VITRO SCREENING ASSAYS FOR COMPOUNDS THAT BIND TO LIVER ACTIVIN

In vitro systems may be designed to identify compounds capable of interacting with (e.g., binding to) liver activin  
20 (including, but not limited to, the mature growth factor domain of liver activin). Compounds identified may be useful, for example, in modulating or disrupting the activity of wild type and/or mutant liver activin gene products or in elaborating the biological function of the liver activin.

25        The principle behind assays that identify compounds that bind to liver activin involves preparing a reaction mixture of liver activin and test compound under conditions that allow the two components to interact and bind, thus forming a complex which can be detected in the reaction mixture and/or  
30 purified. The liver activin species used can vary depending upon the goal of the screening assay. For example, where agonists of natural liver activin are sought, the full length liver activin, or a truncated liver activin, e.g., as described above, can be utilized.

35        The screening assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring the liver activin protein, polypeptide, or

fusion protein onto a solid phase and detecting liver  
activin/test compound complexes anchored on the solid phase  
at the end of the reaction. In one embodiment of such a  
method, the liver activin reactant may be anchored onto a  
5 solid surface, and the test compound, which is not anchored,  
may be labeled, either directly or indirectly.

In practice, microtiter plates may conveniently be  
utilized as the solid phase. The anchored component may be  
immobilized by non-covalent or covalent attachments. Non-  
10 covalent attachment may be accomplished by simply coating the  
solid surface with a solution of the protein and drying.  
Alternatively, an immobilized antibody, preferably a  
monoclonal antibody, specific for the protein to be  
immobilized may be used to anchor the protein to the solid  
15 surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the nonimmobilized  
component is added to the coated surface containing the  
anchored component. After the reaction is complete,  
unreacted components are removed (e.g., by washing) under  
20 conditions such that any complexes formed will remain  
immobilized on the solid surface. The detection of complexes  
anchored on the solid surface can be accomplished in a number  
of ways. Where the previously nonimmobilized component is  
pre-labeled, the detection of label immobilized on the  
25 surface indicates that complexes were formed. Where the  
previously nonimmobilized component is not pre-labeled, an  
indirect label can be used to detect complexes anchored on  
the surface, e.g., using a labeled antibody specific for the  
previously nonimmobilized component (the antibody, in turn,  
30 may be directly labeled or indirectly labeled with a labeled  
anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid  
phase, the reaction products separated from unreacted  
components, and complexes detected, e.g., using an  
35 immobilized antibody specific for liver activin protein,  
polypeptide, derivative or fusion protein or the test  
compound to anchor any complexes formed in solution, and a

labeled antibody specific for the other component of the possible complex to detect anchored complexes.

5           **5.6 METHODS FOR TREATMENT AND REGULATION OF CELL GROWTH  
AND/OR DIFFERENTIATION BY USE OF LIVER ACTIVIN  
ANTIBODIES, ANTAGONISTS, AGONISTS OR GENE PRODUCTS**

The invention encompasses methods and compositions for regulating cell growth and/or differentiation and/or treating cell growth and/or differentiation disorders.

10           Any method which neutralizes or enhances liver activin activity can be used to regulate cell growth and/or differentiation.

For example, the administration of soluble peptides, proteins, fusion proteins, or antibodies (including anti-idiotypic antibodies) that bind to and "neutralize"  
15           circulating liver activin can be used to regulate cell growth and/or differentiation. To this end, peptides corresponding to the extracellular domain of the liver activin receptor, deletion mutants of liver activins, polypeptides or derivatives, or polypeptides containing liver activin mutants  
20           or one or more liver activin domains fused to another polypeptide (e.g., an IgFc polypeptide) can be utilized. Alternatively, anti-idiotypic antibodies or Fab fragments of antiidiotypic antibodies that mimic the liver activin and bind to the activin receptor can be used (see Section 5.3,  
25           supra). Such liver activin proteins, polypeptides, derivatives, fusion proteins, anti-idiotypic antibodies or Fabs are administered to a subject in amounts sufficient to neutralize the interaction between liver activin and liver activin receptor.

30           Liver activin peptides corresponding to the propeptide domain such as, for example, having the amino acid sequence of  $\beta_c$  shown in FIG. 1 from about amino acid residue number 21 to about 236, having the amino acid sequence of  $\beta_e$  shown in FIG. 2 from about amino acid residue number 22 to about 236  
35           may be used or having the amino acid sequence of  $\beta_e$  shown in FIG. 5 from about amino acid residue number 18 to about 236

may be used. Alternatively, liver activin peptides corresponding to the mature growth factor domain, such as, for example, having the amino acid sequence of  $\beta_c$  shown in FIG. 1 from about amino acid residue number 237 to about 354, or having the amino acid sequence of  $\beta_e$  shown in FIG. 2 from about amino acid residue number 237 to about 352 or having the amino acid sequence of  $\beta_e$  shown in FIG. 5 from about 237-350 to about may be used. Additionally, liver activin deletion mutants in which all or part of the propeptide domain and/or mature growth factor domain are missing may also be used. The liver activin receptor, and soluble or bound polypeptides containing all or part of the extracellular domain of the liver activin receptor may also be used. The liver activin protein, polypeptide or derivative and the liver activin receptor proteins or polypeptides may also be fused to heterologous proteins to increase stability and/or to increase or decrease the liver activin activity.

Cell growth and/or differentiation processes which may be regulated according to the methods of the present invention include, but are not limited to, hematopoiesis, erythroid differentiation, ovarian follicular maturation, pituitary growth hormone and adrenocortical tropic hormone secretion, hypothalamic oxytocin secretion, lymphocyte proliferation, neuronal survival, spermatogenesis, bone growth and formation, hepatocyte DNA synthesis, pancreatic insulin secretion, somatostatin induction, cardiac morphogenesis, megakaryocytic differentiation and the induction of mesoderm during early embryonic development.

30

**5.6.1 METHOD OF TREATMENT, REGENERATION, AND GROWTH OF LIVER TISSUE BY USE OF LIVER ACTIVIN GENE PRODUCT ANTIBODY OR ANTAGONIST**

Any method which neutralizes liver activin or inhibits expression of the liver activin gene can be used to enhance the growth or regeneration of liver tissue. The liver activin antibodies and liver activin gene product antagonists

may be used to enhance the growth or regeneration of liver tissue in a variety of situations. In some cases, a patient's liver may be damaged but not beyond repair. For example, and not by way of limitation, excessive consumption of alcohol often leads to cirrhosis of the liver. Hepatocyte destruction can be arrested by discontinuation of alcohol consumption, but recovery may require regeneration of the liver. In such cases, the natural regeneration process may be impaired due to extensive liver damage. In any event, treatment of the patient with pharmaceutical compositions, as described below in Section 5.8, comprising liver activin antibodies or liver activin product antagonists will enhance regeneration and thereby speed recovery.

In some situations, treatment may require transplanting all or a section of the liver of a donor. Regeneration of both a living donor's and a recipient's liver during such transplantation treatments will be aided by administering pharmaceutical compositions, as described below in Section 5.8, comprising a liver activin antibody and/or liver activin gene product antagonist.

In other situations, an artificial liver, produced according to the methods described below, for example, may be implanted into a patient suffering from liver disease. It may be sufficient and desirable to implant such an artificial liver at a stage where it has not yet attained the biological capacity of a normal liver. To increase the capacity of such an implant, the growth rate can be enhanced by administering pharmaceutical compositions, as described below in Section 5.8, comprising liver activin antibody and/or liver activin gene product antagonist.

In cases where a patient's natural liver is damaged or diseased, it may be left intact or only partially removed but still require support from implanted artificial liver tissue or liver tissue transplanted from a donor. Pharmaceutical compositions comprising antibodies or liver activin gene product antagonists can be used in such cases to enhance the

growth of the patient's natural liver tissue, as well as the implanted or transplanted liver tissue.

In vitro liver tissue cultures have a variety of uses. In treating patients suffering from liver damage or disease, for example, the liver tissue cultures can be used to support or replace the natural liver, by direct implantation or as part of an extracorporeal liver device. The regenerative capacity of the liver tissue cultures may be tested using the transgenic mouse system developed by Rhim et al. (1995, Proc. Natl. Acad. Sci. USA 92:4942-4946. In such a system, liver cells are transplanted into the diseased livers of transgenic animals and assessed for their replicative potential.

In addition, such liver tissue cultures can serve as models for testing the toxicity of drugs and other compounds.

Functional in vitro liver tissue may be generated, for example, and not by way of limitation, using the three-dimensional tissue culture system described in U.S. Pat. No. 5,266,480, which is incorporated herein by reference in its entirety. According to this system, hepatocytes may be isolated by conventional methods (Berry and Friend, 1969, J. Cell Biol. 43:506-520) which can be adapted for human liver biopsy or autopsy material. Briefly, a canula is introduced into the portal vein or a portal branch, and the liver is perfused with calcium-free or magnesium-free buffer until the tissue appears pale. The organ is then perfused with a proteolytic enzyme such a collagenase solution at an adequate flow rate. This should digest the connective tissue framework. The liver is then washed in buffer and the cells are dispersed. The cell suspension may be filtered through a 70  $\mu$ m nylon mesh to remove debris. Hepatocytes may be selected from the cell suspension by two or three differential centrifugations.

For perfusion of individual lobes of excised human liver, HEPES buffer may be used. Perfusion of collagenase in HEPES buffer may be accomplished at the rate of about 30 ml/minute. A single cell suspension is obtained by further incubation with collagenase for 15-20 minutes at 37°C

(Guguen-Guillouzo and Guillouzo, eds., 1986, "Isolated and Culture Hepatocytes", Paris, INSERM, and London, John Libbey Eurotext, pp. 1-12; 1982, Cell Biol. Int. Rep. 6:625-628).

The isolated hepatocytes may then be used to inoculate  
5 the three dimensional stroma. The inoculated stroma can be cultured according to the teachings of U.S. Pat. No. 5,266,480, as described for bone marrow and skin, in order to replicate the hepatocytes in vitro, in a system comparable to physiologic conditions. In addition, the growth rate and  
10 development of the three-dimensional liver culture, or of liver tissue cultures grown by alternative methods, may be enhanced by adding liver activin antibodies and/or gene product antagonists to the growth medium. This should result in an increased functional expression by the hepatocytes.

15

**5.6.2 STIMULATION OF HEMATOPOIESIS  
BY USE OF LIVER ACTIVIN GENE PRODUCT**

Any method which elevates liver activin concentration and/or activity can be used to stimulate hematopoiesis.  
20 Using these methods, the liver activin gene and nucleotide sequences described in Section 5.1 and proteins, polypeptides, and derivatives described in Section 5.2, above may be used to stimulate hematopoiesis.

According to the invention, the liver activin  
25 compositions described herein, therapies directed towards elevating the concentration of blood cells such as hematopoietin therapy. In a specific embodiment, the liver activin compositions described herein are used in erythropoietin therapy, which is directed toward  
30 supplementing the oxygen carrying capacity of blood. Liver activin treatment within the scope of the invention includes but is not limited to patients generally requiring blood transfusions, such as, for example, trauma victims, surgical patients, dialysis patients, and patients with a variety of  
35 blood composition-affecting disorders, such as hemophilia, cystic fibrosis, pregnancy, menstrual disorders, early anemia of prematurity, spinal cord injury, space flight, aging,

various neoplastic disease states, and the like. Examples of patient conditions that require supplementation of the oxygen carrying capacity of blood and which are within the scope of this invention, include but are not limited to: treatment of  
5 blood disorders characterized by low or defective red blood cell production, anemia associated with chronic renal failure, stimulation of reticulocyte response, development of ferrokinetic effects (such as plasma iron turnover effects and marrow transit time effects), erythrocyte mass changes,  
10 stimulation of hemoglobin C synthesis, and increasing levels of hematocrit in vertebrates. The invention also provides for treatment to enhance the oxygen-carrying capacity of an individual, such as for example, an individual encountering hypoxic environmental conditions.

15 The invention also encompasses combining the liver activin compositions described in Sections 5.1 and 5.2 with other proposed or conventional hematopoietic therapies. Thus, for example, the liver activin can be combined with compounds that singly exhibit erythropoietic stimulatory  
20 effects, such as erythropoietin, testosterone, progenitor cell stimulators, insulin-like growth factor, prostaglandins, serotonin, cyclic AMP, prolactin, and triiodothyronine. Also encompassed are combinations with compounds generally used to treat aplastic anemia, such as methenolone, stanozolol, and  
25 nandrolone; to treat iron-deficiency anemia, such as iron preparations; to treat malignant anemia, such as vitamin B<sub>12</sub> and/or folic acid; and to treat hemolytic anemia, such as adrenocortical steroids, e.g., corticoids. See e.g., Resegotti et al., 1981, Panminerva Medica, 23:243-248; Kurtz,  
30 1982, FEBS Letters, 14a:105-108; McGonigle et al., 1984, Kidney Int., 25:437-444; and Pavlovic-Kanter, 1980, Expt. Hematol., 8(supp. 8) 283-291.

Compounds that enhance the effects of or synergize with erythropoietin are also useful as adjuvants herein, and  
35 include but are not limited to, adrenergic agonists, thyroid hormones, androgens, hepatic erythropoietic factors, erythrotropins, and erythroginins, See for e.g., Dunn,

- "Current Concepts in Erythropoiesis", John Wiley and Sons (Chichester, England, 1983); Weiland et al., 1982, Blut, 44:173-175; Kalmani, 1982, Kidney Int., 22:383-391; Shahidi, 1973, New Eng. J. Med., 289:72-80; Urabe et al., 1979, J. Exp. Med., 149:1314-1325; Billat et al., 1982, Expt. Hematol., 10:133-140; Naughton et al., 1983, Acta Haemat, 69:171-179; Cognote et al. in abstract 364, Proceedings 7th Intl. Cong. of Endocrinology (Quebec City, Quebec, July 1-7, 1984); and Rothman et al., 1982, J. Surg. Oncol., 20:105-108.
- 10       Methods for stimulating hematopoiesis comprise administering a hematopoietically effective amount (i.e, an amount which effects the formation of blood cells) of a pharmaceutical composition containing liver activin to a patient. The liver activin is administered to the patient by
- 15 any suitable technique, including but not limited to, parenteral, sublingual, topical, intrapulmonary and intranasal, and those techniques further discussed in Section 5.8. The pharmaceutical composition optionally contains one or more members of the group consisting of erythropoietin,
- 20 testosterone, progenitor cell stimulators, insulin-like growth factor, prostaglandins, serotonin, cyclic AMP, prolactin, triiodothyronine, methenolene, stanozolol, and nandrolone, iron preparations, vitamin B<sub>12</sub>, folic acid and/or adrenocortical steroids.
- 25       The liver activin and cotreatment drug(s) are suitably delivered by separate or by the same administration route, and at the same or at different times, depending, e.g., on dosing, the clinical condition of the patient, etc.

30                   5.6.3       **METHOD OF STIMULATING BONE GROWTH  
BY USE OF LIVER ACTIVIN GENE PRODUCT**

- Any method which elevates liver activin concentration and/or activity can be used to effectuate bone growth (i.e., bone mass). Such approaches can be used to treat bone
- 35 fractures, defects, and disorders which result in weakened bones such as osteoporosis, osteomalacia, and age-related loss of bone mass. According to the invention, bone growth

is enhanced by local and/or systemic administration of a pharmaceutical composition of liver activin in an osteogenically effective amount (i.e., an amount which effects the formation and development of mature bone).

- 5       The compositions of the invention described in Section 5.1 and Section 5.2 above, may optionally contain osteogenically effective amounts of other bone growth promoting compounds, including beta-type transforming growth factors ("TGF- $\beta$ s") (e.g., TGF- $\beta$  1,-2,-3, proteins, 10 polypeptides, derivatives (including fragments), and homologous proteins thereof) and/or bone morphogenic proteins ("BMPs") (e.g., BMP-, -2,-3,-4,-5,-6, or -7 proteins, polypeptides, and homologous proteins thereof) and/or osteogenic proteins (including polypeptides, and homologous 15 proteins thereof) and/or parathyroid hormone. BMPs and TGF- $\beta$ s may be prepared by methods known in the art (see e.g., PCT/US87/01537 and 4,774,322 which are incorporated herein by reference in their entirety). Alternatively, TGF- $\beta$ s are available from commercial sources (R&D Systems, Minneapolis, 20 Minn.).

In an alternative embodiment, the liver activin compositions of the invention include other therapeutics such as for example, in the case of treatment of osteoporosis, fluoride, calcitonin, vitamin D metabolites, estrogen, and 25 parathyroid hormone.

Methods for inducing local bone growth include administering an osteogenically effective amount of liver activin, optionally in combination with TGF- $\beta$ , BMP, bone marrow, or proteins extracted therefrom, in a 30 pharmaceutically acceptable carrier.

One embodiment of the invention encompasses a method for inducing systemic bone growth, comprising administering an osteogenically effective amount of liver activin optionally including TGF- $\beta$ , BMP, bone marrow, or proteins extracted 35 therefrom, in a pharmaceutically acceptable carrier.

Another embodiment provides a method for regulating bone growth which comprises initially administering an effective

amount of TGF- $\beta$  and BMPs and sequentially administering an effective amount of activin. Preferably, to induce, endochondral bone growth, combinations of BMP, TGF- $\beta$ , may be locally administered to induce cartilage modeling. Activin  
5 may be subsequently administered subcutaneously and/or systemically to induce mature bone formation and differentiation of the cartilage model induced by TGF- $\beta$  and BMP. According to this method, the activin would enhance mineralization of the endochondral bone. It is expected that  
10 the quantity of mature bone formed by this method would be greater than the quantity of mature bone formed by either TGF- $\beta$ , BMPs, or liver activin alone, or combinations of TGF- $\beta$  and BMPs, or combinations of liver activin and BMPs.

Alternative, nonlimiting methods for regulating bone  
15 growth and maturation comprises locally administering an osteogenically effective amount of activin with TGF- $\beta$ , BMP, and/or bone marrow or proteins extracted therefrom to induce bone growth, followed by systemically administration of an osteogenically effective amount of liver activin.

20

#### **5.7 METHODS FOR REGULATING LIVER ACTIVIN ACTIVITY AND/OR TREATMENT OF LIVER DISEASE BY AFFECTING LIVER ACTIVIN GENE EXPRESSION**

Any method which inhibits or activates expression of the liver activin gene (either transcription or translation) can  
25 be used to regulate cell growth and/or differentiation activities affected by liver activin genes. Described below are methods whereby cell growth and/or differentiation may be regulated and cell growth and/or differentiation disorders may be treated with the nucleic acid sequences described in  
30 Section 5.1, above.

In certain cases, an increase in liver activin activity may be desired to facilitate liver activin regulated mechanisms, such as, for example, to stimulate liver  
regeneration and/or bone growth. Furthermore, certain cell  
35 growth and/or differentiation disorders may be brought about, at least in part, by the absence or reduction of the level of

liver activin gene expression. As such, an increase in the level of gene expression would bring about the amelioration of symptoms relating to this disorder. Techniques for increasing liver activin gene expression levels are discussed 5 in Section 5.7.1, below.

In some cases, the reduction in the level and/or activity of liver activin gene products would decrease or prevent the inhibitory effect of liver activin, and it would therefore be desirable to reduce liver activin gene product. 10 Techniques for the reduction of liver activin, gene expression levels are discussed in Section 5.7.2, below.

#### 5.7.1 RESTORATION OR INCREASE IN LIVER ACTIVIN EXPRESSION OR ACTIVITY

15 With respect to an increase in the level of normal liver activin gene expression and/or liver activin gene product activity, liver activin nucleic acid sequences can be utilized for the regulation of cell growth and/or differentiation and/or treatment of cell growth and/or 20 differentiation disorders. Treatment can be administered, for example, in the form of gene replacement therapy. Specifically, one or more copies of a normal liver activin gene or a portion of the liver activin gene that directs the production of a liver activin gene product exhibiting normal 25 function, may be inserted into the appropriate cells within a patient or animal subject, using vectors which include, but are not limited to, adenovirus, adeno-associated virus, retrovirus and herpes virus vectors, in addition to other particles that introduce DNA into cells, such as liposomes and gene activated matrices. Methods of transferring nucleic 30 acids into mammalian repair cells using gene activated matrices is further described in U.S. application Serial No. 08/631,334, filed April 12, 1996, the contents of which is hereby incorporated by reference in its entirety.

35 Because the liver activin gene is expressed in the liver, such gene replacement therapy techniques should be capable of delivering liver activin gene sequences to cells

in this tissue within patients, or, alternatively, should involve direct administration of such liver activin gene sequences to the site of the cells in which the liver activin gene sequences are to be expressed. Alternatively, targeted homologous recombination can be utilized to correct the defective endogenous liver activin gene in the appropriate tissue; e.g., liver.

Additional methods which may be utilized to increase the overall level of liver activin gene expression and/or liver  
10 activin activity include the introduction of appropriate liver activin-expressing cells, preferably autologous cells, into a patient at positions and in numbers which are sufficient to ameliorate the symptoms of cell growth and/or differentiation disorders. Such cells may be either  
15 recombinant or non-recombinant. Among the cells which can be administered to increase the overall level of liver activin gene expression in a patient are normal cells, which express the liver activin gene. The cells can be administered at the anatomical site in the liver, or as part of a tissue graft  
20 located at a different site in the body. Such cell-based gene therapy techniques are well known to those skilled in the art, see, e.g., Anderson, et al., U.S. Patent No. 5,399,349; Mulligan & Wilson, U.S. Patent No. 5,460,959.

**25                      5.7.2        METHODS FOR DECREASING  
LIVER ACTIVIN EXPRESSION OR ACTIVITY**

A variety of techniques may be utilized to reduce or inhibit the expression of liver activin genes using molecules derived from the sequences described in Section 5.1, above. The level of endogenous liver activin gene expression can be reduced, e.g., using antisense or ribozyme approaches to inhibit or prevent translation of the liver activin mRNA transcripts; triple helix approaches to inhibit transcription of the liver activin gene; or targeted homologous recombination to inactivate or "knock out" the liver activin gene or its endogenous promoter. One or more copies of a nucleotide sequence may be inserted into the appropriate

cells within a patient or animal subject, using, for example but not by way of limitation, adenovirus, adeno-associated virus, retrovirus and herpes virus vectors, in addition to other particles that introduce nucleic acids into cells, such as liposomes and gene activated matrices.

Because the liver activin gene is expressed in the adult liver, delivery techniques should be preferably designed to target this tissue. Alternatively, the antisense, ribozyme or DNA constructs described herein could be administered directly to the site containing the target cells; e.g., the adult liver.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to liver activin mRNA. The antisense oligonucleotides will bind complementary liver activin mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently shown to be effective at inhibiting translation of mRNAs as well. See, generally, Wagner, R., 1994, Nature

372:333-335. Thus, oligonucleotides complementary to either the 5'- or 3'- non- translated, non-coding regions of the liver activin genes could be used in an antisense approach to inhibit translation of endogenous liver activin mRNA.

- 5 Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether  
10 designed to hybridize to the 5'-, 3'- or coding region of liver activin mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least  
15 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that in vitro studies are first performed to quantitate the ability of the antisense oligonucleotide to  
20 inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an  
25 internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the  
30 test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric  
35 mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate

backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may also be conjugated with other appended groups such as peptides (e.g., for targeting host cell receptors in vivo); agents  
5 facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT  
10 Publication No. WO89/10134, published April 25, 1988); hybridization-triggered cleavage agents (See, e.g., Krol et al., 1988, BioTechniques 6:958-976); and intercalating agents (see, e.g., Zon, 1988, Pharm. Res. 5:539-549).

The antisense oligonucleotide may comprise at least one  
15 modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine,  
20 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine,  
25 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil,  
30 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least  
35 one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a  
5 phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an  $\alpha$ -anomeric oligonucleotide. An  $\alpha$ -anomeric oligonucleotide forms specific double-stranded hybrids with  
10 complementary RNA, in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al.,  
15 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples,  
20 phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

25 While antisense nucleotides complementary to the liver activin coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred. For example, antisense oligonucleotides having the following sequences can be utilized in accordance with  
30 the invention:

- a) 5'- CATTGCTGGAGGATAGCC-3' which is complementary to nucleotides 134 to 151 in FIG. 1.
- 35 b) 5'- CATTGCTGGAGGATAGCCTCCTT -3' which is complementary to nucleotides 129 to 151 in FIG. 1.

- c) 5'- CATTGCTGGAGGATAGCCTCCTTAAAGGA -3' which is complementary to nucleotides 123 to 151 in FIG. 1.
- 5 d) 5'- CATTGCTGGAGGATAGCCTCCTTAAAGGACCCAAA1-3' which is complementary to nucleotides 117 to 151 in FIG. 1.
- e) 5'- TGCTGGAGGATAGCCTCCTTAAAGGACCC -3' which is complementary to nucleotides 120 to 148 in FIG. 1.
- 10 f) 5'- AGGATAGCCTCCTTAAAGGACCC -3' which is complementary to nucleotides 120 to 142 in FIG. 1.
- g) 5'- GCCTCCTTAAAGGACCC -3' which is complementary to nucleotides 120 to 136 in FIG. 1.
- 15 h) 5'- CATGCTCCAGGTAGATG -3' which is complementary to nucleotides 202 to 218 in FIG. 2.
- i) 5'- CATGCTCCAGGTAGATGGCTCTG -3' which is complementary to nucleotides 196 to 218 in FIG. 2.
- 20 j) 5'- CATGCTCCAGGTAGATGGCTCTGCTTTGA -3' which is complementary to nucleotides 190 to 218 in FIG. 2.
- 25 k) 5'- CATGCTCCAGGTAGATGGCTCTGCTTTGAGAACCT -3' which is complementary to nucleotides 184 to 218 in FIG. 2.
- l) 5'- CTTGATGCTCCAGGTAGATGGCTCTGCTTTGAGAA -3' which is complementary to nucleotides 187 to 221 in FIG. 2.
- 30 m) 5'- GCTCCAGGTAGATGGCTCTGCTTTGAGAA -3' which is complementary to nucleotides 187 to 215 in FIG. 2.
- n) 5'- GGTAGATGGCTCTGCTTTGAGAA -3' which is complementary to nucleotides 187 to 209 in FIG. 2.
- 35

- o) 5'- TGGCTCTGCTTTGAGAA -3' which is complementary to nucleotides 187 to 203 in FIG. 2.
- 5 p) 5'- TGCTTTGAGAA -3' which is complementary to nucleotides 187 to 197 in FIG. 2.
- q) 5'- CATGCTCCTGGTGAATGG -3' which is complementary to nucleotides 60 to 78 in FIG. 5.
- 10 r) 5'- CATGCTCCTGGTGAATGGTGGTT -3' which is complementary to nucleotides 55 to 78 in FIG. 5.
- s) 5'- CATGCTCCTGGTGAATGGTGGTTCAGGAG -3' which is complementary to nucleotides 49 to 78 in FIG. 5.
- 15 t) 5'- CATGCTCCTGGTGAATGGTGGTTCAGGAG -3' which is complementary to nucleotides 43 to 78 in FIG. 5.
- u) 5'- GCTCCTGGTGAATGGTGGTTCAG -3' which is complementary to nucleotides 47 to 75 in FIG. 5.
- 20 v) 5'- CCTGGTGAATGGTGGTT -3' which is complementary to nucleotides 44 to 71 in FIG. 5.
- 25 w) 5'- GGTGAATGGTG -3' which is complementary to nucleotides 41 to 68 in FIG. 5.

The antisense molecules should be delivered to cells which express the liver activin in vivo, e.g., hepatocytes.

30 A number of methods have been developed for delivering antisense DNA or RNA to cells, e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that

35 specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

It is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the  
5 antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that form complementary base pairs with the  
10 endogenous liver activin transcripts and thereby prevent translation of the liver activin mRNA. For example, a vector can be introduced in vivo such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally  
15 integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells.  
20 Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981,  
25 Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene  
30 (Brinster et al., 1982, Nature 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site; e.g., the liver. Alternatively, viral vectors can be used which selectively  
35 infect the desired tissue; (e.g., for brain, herpesvirus vectors may be used), in which case administration may be accomplished by another route (e.g., systemically).

Ribozyme molecules designed to catalytically cleave liver activin mRNA transcripts can also be used to prevent translation of liver activin mRNA and expression of liver activin. (See, e.g., PCT International Publication 5 WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy liver activin mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations 10 dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in 15 Haseloff and Gerlach, 1988, Nature, 334:585-591. For example, there are over 110 and over 80 potential hammerhead ribozyme cleavage sites within the nucleotide sequence of murine  $\beta_c$  and  $\beta_e$ , respectively (FIG. 1 and FIG. 2). Preferably the ribozyme is engineered so that the cleavage 20 recognition site is located near the 5' end of the liver activin mRNA, i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

For example, hammerhead ribozymes having the following 25 sequences can be utilized in accordance with the invention:

- a) 5'-UGGAGGAUAGCCUCCUUAAGGACCCAAAGCAGGNNNNCCUGAGNA  
GUCAGCCUAUAUUCAGGGUCUCA-3' which will cleave between  
nucleotides 117 and 118 in FIG. 1.
- 30 b) 5'-GGAGCAAGGAGGAGGCCAAAGCCGNNNNCCGGAGNA  
GUCUGCUGGAGGAUAGCCUCCUUA-3' which will cleave between  
nucleotides 148 and 149 in FIG. 1.
- 35 c) 5'-GUUCACUACUGUGGUUGGAGUCAGGAACAAAGCUGUNNNNA  
CAGAGNAGUCAAGAGCCAGGAGCAAGGA-3' which will cleave between  
nucleotides 175 and 176 in FIG. 1.

- d) 5'-ACAUGAAGCGGGUCUGCCGGACCUCCAAAGCUGANNNNUCAGAGNA  
GUCGCCACUGGCCAUUCUACCAGAG-3' which will cleave between  
nucleotides 526 and 527 in FIG. 1.
- 5 e) 5'-UUCUGGUACCAAAGCGGGNNNNCCCGAGNA  
GUCCUCCAGAGUAAGGUG-3' which will cleave between nucleotides  
742 and 743 in FIG. 1.
- f) 5'-AACCUCGAAUGGGUGGCAAAGCCGCNNNNNGCGGAGNA  
10 GUCUUUUAUGAGUCUGAUUU-3' which will cleave between nucleotides  
169 and 170 in FIG. 2.
- g) 5'-UGGAAGCUUCAUGCUCCAAAGCAGANNNNUCUGAGNA  
GUCGUAGAUGGCUCUG-3' which will cleave between nucleotides 208  
15 and 209 in FIG. 2.
- h) 5'-UGCCCACAGCAGUAUUAGCCAAAGCGGUNNNNACCGAGNA  
GUCAGCUGGGGUUUUGGAAGCUUCAU-3' which will cleave between  
nucleotides 238 and 239 in FIG. 2.
- 20 i) 5'-UUGGGCCCCCACAGGACGGGCAAAGCCGANNNNUCGGAGNA  
GUCGCAGAUUCUACUCUGCACCC-3' which will cleave between  
nucleotides 289 and 290 in FIG. 2.
- 25 j) 5'-CGGCUGCAGGAUCCAAUCCCGCCACCCCAAAGCGGCNNNNGCCG  
AGNAGUCCUCCUGGAAGUCUAC-3' which will cleave between  
nucleotides 989 and 990 in FIG. 2.
- k) 5'-CTTGACCACATTGCCAAAGCGGUNNNNACCGAGNA  
30 GUCUAUGGUCAAGGUAGAGGAG-3' which will cleave between  
nucleotides 1206 and 1207 in FIG. 2.
- l) 5'-CCGCAAAGCAGANNNNUCUGAGNAGUCGCUCCU -3' which will  
cleave between nucleotides 75 and 76 in FIG. 5.
- 35 m) 5'-GGACAAAGCAGANNNNUCUGAGNAGUCCAGGGA -3' which will  
cleave between nucleotides 88 and 89 in FIG. 5.

n) 5'-UCGCAAAGCAGANNNNUCUGAGNAGUCCAGUGC -3' which will cleave between nucleotides 123 and 124 in FIG. 5.

o) 5'-ACACAAAGCAGANNNNUCUGAGNACUACCCUG -3' which will cleave between nucleotides 139 and 140 in FIG. 5.

p) 5'-CCACAAAGCAGANNNNUCUGAGNAGUCGAGGGA -3' which will cleave between nucleotides 158 and 159 in FIG. 5.

The ribozymes of the present invention also include RNA  
10 endoribonucleases (hereinafter "Cech-type ribozymes"), such as the one which occurs naturally in Tetrahymena thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224:574-578; Zaug and Cech, 1986,  
15 Science, 231:470-475; Zaug, et al., 1986, Nature, 324:429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA  
20 sequence, whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the liver activin RNA.

As in the antisense approach, the ribozymes can be  
25 composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express the liver activin in vivo. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III  
30 or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous liver activin messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

35 Endogenous liver activin gene expression can also be reduced by inactivating or "knocking out" the liver activin gene or its promoter using targeted homologous recombination.

(E.g., see Smithies et al., 1985, Nature 317:230-234; Thomas & Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989 Cell 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional  
5 liver activin (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous liver activin gene (either the coding regions or regulatory regions of the liver activin gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect  
10 cells that express liver activin in vivo. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the liver activin gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to  
15 generate animal offspring with an inactive liver activin (e.g., see Thomas & Capecchi 1987 and Thompson 1989, supra). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site in vivo using  
20 appropriate viral vectors.

Alternatively, endogenous liver activin gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the liver activin gene (i.e., the liver activin promoter and/or enhancers) to  
25 form triple helical structures that prevent transcription of the liver activin gene in target cells in the body. (See generally, Helene, C. 1991, Anticancer Drug Des., 6(6):569-84; Helene, C., et al., 1992, Ann, N.Y. Acad. Sci., 660:27-36; and Maher, L.J., 1992, Bioassays 14(12):807-15).

30 In yet another embodiment of the invention, the activity of liver activin can be reduced using a "dominant negative." To this end, constructs which encode defective liver activin, such as, for example, mutants lacking all or a portion of the endoproteolytic motif, and/or mature growth factor domain,  
35 can be used in gene therapy approaches to diminish the activity of the liver activin on appropriate target cells. For example, nucleotide sequences that direct host cell

expression of liver activin in which all or a portion of the propeptide domain, endoproteolytic motif, and/or mature growth factor domain, is altered or missing can be introduced into cells in the liver (either by in vivo or ex vivo gene therapy methods described above). Alternatively, targeted homologous recombination can be utilized to introduce such deletions or mutations into the subject's endogenous liver activin gene in the liver. The engineered cells will express non-functional liver activin (i.e., a ligand that may be capable of binding, but which is incapable of inducing signal transduction).

#### 5.8 PHARMACEUTICAL PREPARATIONS AND METHODS OF ADMINISTRATION

The liver activin proteins and polypeptides as well as compounds that affect liver activin gene expression or gene product activity can be administered to a patient at therapeutically effective doses to regulate, treat or ameliorate cell growth and/or differentiation disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in regulation and/or amelioration of symptoms of cell growth and/or differentiation disorders.

##### 5.8.1 EFFECTIVE DOSE

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of

affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating dosage range for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the  $IC_{50}$  (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

20

#### 5.8.2 FORMULATIONS AND USE

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration. Administration can be systemic or local.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate);

lubricants (e.g., magnesium stearate, talc or silica);  
disintegrants (e.g., potato starch or sodium starch  
glycolate); or wetting agents (e.g., sodium lauryl sulphate).  
The tablets may be coated by methods well known in the art.

5 Liquid preparations for oral administration may take the form  
of, for example, solutions, syrups or suspensions, or they  
may be presented as a dry product for constitution with water  
or other suitable vehicle before use. Such liquid  
preparations may be prepared by conventional means with

10 pharmaceutically acceptable additives such as suspending  
agents (e.g., sorbitol syrup, cellulose derivatives or  
hydrogenated edible fats); emulsifying agents (e.g., lecithin  
or acacia); non-aqueous vehicles (e.g., almond oil, oily  
esters, ethyl alcohol or fractionated vegetable oils) and

15 preservatives (e.g., methyl or propyl-p-hydroxybenzoates or  
sorbic acid). The preparations may also contain buffer salts  
and flavoring-, coloring- and sweetening- agents as  
appropriate.

Preparations for oral administration may be suitably  
20 formulated to give controlled release of the active compound.

For buccal administration the compositions may take the  
form of tablets or lozenges formulated in conventional  
manner.

For administration by inhalation, the compounds for use  
25 according to the present invention are conveniently delivered  
in the form of an aerosol spray from pressurized packs or a  
nebulizer, with the use of a suitable propellant, e.g.,  
dichlorodifluoromethane, trichlorofluoromethane,  
dichlorotetrafluoroethane, carbon dioxide or other suitable

30 gas. In the case of a pressurized aerosol. the dosage unit  
may be determined by providing a valve to deliver a metered  
amount. Capsules and cartridges of, e.g., gelatin for use in  
an inhaler or insufflator. may be formulated containing a  
powder mix of the compound and a suitable powder base such as

35 lactose or starch.

The compounds may be formulated for parenteral  
administration by injection, e.g., by bolus injection or

continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or  
5 emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. Further  
10 discussion of vehicles for parenteral administration may be found in E.W. Martin, "Remington's Pharmaceutical Sciences" (Mack Pub. Co.).

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g.,  
15 containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by  
20 implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly  
25 soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack, may  
30 for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

In one embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the  
35 area in need of treatment. This may be achieved, for example, and not by way of limitation, by local infusion during surgery; topical application, e.g., in conjunction

- with a wound dressing after surgery; injection, from a catheter, with a suppository, or from an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In a specific embodiment, the porous end of dental or orthopedic implants, which is inserted into the implant site, may be coated with osteoinductive proteins such as liver activin, activin A, activin B, BMP, and TGF- $\beta$ , to enhance attachment of the implant device to the bone.
- 10 Alternatively, the implants may be coated with DNA encoding osteoinductive proteins such as liver activin, activin A, activin B, BMP and TGF- $\beta$ .

6. EXAMPLE: IDENTIFICATION AND CHARACTERIZATION OF THE ACTIVIN/INHIBIN  $\beta_c$  AND  $\beta_e$  GENE

- 15 The identification and characterization of activin  $\beta_c$  and  $\beta_e$  subunits, two members of a novel subgroup of activins, is described herein. cDNA clones encoding two members of the transforming growth factor (TGF- $\beta$ ) superfamily have been isolated and characterized in the mouse. Both cDNAs appear to code for activin  $\beta$  subunits—i.e., one gene is likely the mouse homolog of human activin  $\beta_c$ , while the second is novel and has been designated activin  $\beta_e$ . In addition, a human activin  $\beta_e$  cDNA clone has been identified and characterized.
- 20 Our studies suggest that in certain respects activin  $\beta_c$  and  $\beta_e$  are more like one another than other known activins: (a), these genes are physically linked, with only 5-kbp of genomic DNA separating the 3' end of activin  $\beta_c$  from the 5' end of activin  $\beta_e$ ; (b), the organization of both genetic loci is similar, consisting of two coding exons separated by a single intervening sequence; (c), the predicted carboxy-terminal sequences of both activins are highly homologous, with 62% amino acid identity over the mature growth factor region; (d), both genes appear to be specifically and highly expressed in adult mouse liver and (e) the results of several
- 30 in vitro assays show that homodimers of activin  $\beta_c$  and  $\beta_e$  stimulate the proliferation of HepG2 cells. When taken

together, our data suggest that the activin  $\beta_c$  and  $\beta_e$  subunit genes may have evolved from a common ancestor that underwent a tandem duplication. These structural and functional data differentiate activin  $\beta_c$  and  $\beta_e$  subunits from other known  
5 activins, suggesting that these subunits represent a unique subgroup of TGF- $\beta$ -like molecules.

### 6.1 MATERIALS AND METHODS

PCR. Genomic DNA templates were isolated from NIH3T3  
10 cells using standard methods (Sambrook et al., 1989, supra). RNA and cDNA templates were prepared as described by Chen et al. (1993, J. Biol. Chem., 268:27,381-27,389). Primer sequences were as follows: upstream primer (forward), 5' GT(ACGT) GG(ACGT) TGG AA(CT) GA(CT) TGG AT 3'; and downstream  
15 primer (reverse complement), 5' CA (ACGT)CC (AG)CA (ACGT)CC (CT)TC (ACGT)AC (ACGT)AC 3'. Note that the individual codons have been separated by a space and that degeneracy was achieved by adding a cocktail of nucleotides during  
20 synthesis, as shown in parentheses, at selected wobble positions. For both genomic DNA and cDNA templates, PCR proceeded through 35 cycles of denaturation (94°C, 1.5 min), annealing (50°C, 1 min, 10 sec), and elongation (72°C, 1 min, 10 sec).

cDNA Cloning. Aliquots of 50,000 plaque forming  
25 units/plate (Mouse Liver 5'-Stretch Plus cDNA library in Lambda gt11, from adult BALB/c male mice, Clontech Laboratories, Inc., Palo Alto, CA) and fresh overnight Y1088 cells (grown in LB medium supplemented with 0.2% maltose in 10mM MgSO<sub>4</sub>) were mixed, incubated for 15 min at 37°C, mixed  
30 again with 8 ml of liquid top layer agarose consisting of NZY Broth (Gibco-BRL Life Technologies Inc., Grand Island, NY) plus 0.75% agarose, and then spread evenly onto freshly poured 150 mm NZY-agar plates. Standard methods were used to prepare plaque-lifts and perform filter hybridization (42°C,  
35 in 50% formamide, 5 x SSC buffer, 1 x Denhardt's solution, 10 mM Tris, pH 7.5, 0.1% SDS, and 100  $\mu$ g/ml salmon sperm DNA). Sambrook et al., 1989, supra. Filters were washed

progressively to high stringency (0.1 x SSC buffer, 0.1% SDS, 65°C). cDNA probes were radiolabelled by the random priming method using commercially available reagents and protocols (Random Primed DNA Labeling Kit, Amersham Life Sciences, Inc., Arlington Heights, IL). Purified phage clones were cultured with Y1088 cells, and phage DNA was extracted using the protocols and reagents with the Wizard™ Lambda Prep kit (Promega, Madison, WI). Phage DNA was digested with EcoRI, the recombinant insert was purified and subcloned into pBluescript plasmid vector (Stratagene, La Jolla, CA) using standard protocols, and the insert was sequenced using Sequenase (version 2.0, United States Biochemical Company, Cleveland, OH) according to the manufacturer's protocols. Sequence alignment and identity were determined using software from Genetics Computer Group, Madison, WI (GrowTree™, version 8.1, UPMGA method of analysis).

#### Isolation and Characterization of Mouse Genomic Clones.

A genomic library in the Lambda Fix II vector (made from mouse strain 129SVJ liver DNA, Stratagene) was plated at 50,000 plaques/plate, and 20 nitrocellulose replicas were screened at high stringency as described above using a labeled  $\beta_c$  cDNA as the probe. Filters were washed progressively to high stringency also as described above and autoradiographed. Positive phage plaques were re-screened until purified, and DNA was prepared as described in the Wizard™ Lambda Pre kit (Promega). Five unique clones from a pool of ten isolates, each with more than 10-kbp of genomic DNA, were isolated. NotI digests of all unique clones were subcloned into NotI digested pBluescript plasmid vector (Stratagene). Genomic inserts were characterized by Southern analysis and by DNA sequencing.

#### Isolation and Characterization of a Human Activin $\beta_e$ Clone.

The mouse activin  $\beta_e$  plasmid pbE #2 was used as a probe to screen a cDNA library. Aliquots of 50,000 plaque forming units/plate (Human Liver 5'-Stretch Plus cDNA library in Lambda gt11, from adult human liver, Clontech Laboratories, Inc., Palo Alto, CA) and fresh overnight Y1088

cells (grown in LB medium supplemented with 0.2% maltose in 10 mM MgSO<sub>4</sub>) were mixed, incubated for 15 min at 37°C, mixed again with 8 ml of liquid top layer agarose consisting of NZY Broth (Gibco-BRL Life Technologies Inc.,  
5 Grand Island, NY) plus 0.75% agarose, and then spread evenly onto freshly poured 150 mm NZY-agar plates. Standard methods were used to prepare plaque-lifts and perform filter hybridization (37°C, in 50% formamide, 5xSSC buffer, 1 x Denhardt's solution, 10 mM Tris, pH 7.5, 0.1% SDS, and 100  
10 µg/ml salmon sperm DNA). Filters were washed progressively to low stringency (0.5 x SSC buffer, 0.1% SDS, 55°C). The cDNA probe was radiolabeled by the random priming method using commercially available reagents and protocols (Random Primed DNA Labeling Kit, Amersham Life Sciences, Inc.,  
15 Arlington Heights, IL). Purified phage clones were cultured with Y1088 cells, and phage DNA was extracted using the protocols and reagents with the Wizard<sup>SM</sup> Lambda Prep kit (Promega, Madison, WI). Phage DNA was digested with Eco RI, the recombinant insert was purified and subcloned into a  
20 pBluescript plasmid vector (Stratagene, La Jolla, CA) using standard protocols, and the insert was sequenced using Sequenase (version 2.0, United States Biochemical Company, Cleveland, OH) according to the manufacturer's protocols. Sequence alignment and identity were determined using  
25 software from the Genetics Computer Group, Madison, WI (GrowTree<sup>SM</sup>, version 8.1, UPMGS method of analysis).

Northern analysis. An adult mouse multiple tissue Northern blot was purchased from Clontech. According to product information, each lane of the blot contains about 2  
30 µg of poly(A) RNA. Specific cDNA probes, consisting solely of untranslated sequence from the 3' untranslated region of activin  $\beta_c$  (nucleotides 1230-1555) and  $\beta_e$  (nucleotides 1822-2130), were generated by subcloning and then <sup>32</sup>P-labeled by random priming and used for hybridization (42°C, overnight)  
35 in 50% formamide, 5 x SSPE buffer (pH 7.4, BioWhittaker, Walkersville, MD), 10 x Denhardt's, 2% SDS, 100 µg/ml salmon sperm DNA. Blots were washed progressively from

hybridization buffer to 0.1 x SSC, 0.1% SDS (55°C), and then placed against x-ray film (X-Omat AR film, Eastman Kodak Co., Rochester, NY) with intensifying screens at -86°C for the indicated times.

- 5        Expression Plasmids. In order to screen for function, expression plasmids for activin  $\beta_C$ ,  $\beta_E$ , and  $\beta_A$  subunits were prepared as described below. A hemagglutinin ("HA") epitope tag (Fang et al., 1996, Proc. Natl. Acad. Sci. U.S.A., 93:5753-5758) was added to the coding sequence of each
- 10 expression plasmid. To insure the integrity of the final construct, all three expression plasmids were subjected to restriction mapping and DNA sequence analysis.

An expression plasmid coding for the mouse activin  $\beta_C$  subunit was assembled into the pcDNA3 vector (Invitrogen Corp., San Diego, CA). Prior to subcloning, the HA epitope was added to the 3' end of the activin  $\beta_C$  coding sequence by PCR. Primer sequences were as follows: upstream primer (forward), 5' CCG AAT TCC ATG GCC TCC TTG CTC CTG GC 3', and downstream primer (reverse complement), 5' GGG AAT TCA AGC

20 GTA ATC CGG AAC GTA ACT ACA CCC GCA GGC CTC GAC CAC GGG ATC C 3'. Note that the downstream primer has been designed so that the PCR product codes for the HA epitope and has an EcoRI site at its 3' end. Plasmid from a cDNA clone containing the entire  $\beta_C$  coding region was used as a source of

25 template DNA. PCR proceeded through 25 cycles of denaturation (94°C, 1.0 min), annealing (65°C, 1 min, 10 sec), and elongation (72°C, 1 min, 10 sec). The PCR product (1.1-kbp) was digested with EcoRI, purified by agarose gel electrophoresis, and subcloned into the pcDNA3 vector.

- 30        An expression plasmid coding for the mouse activin  $\beta_E$  subunit was also assembled into the pcDNA3 vector. An HA epitope was added to the 3' end of the coding sequence essentially as described above. Primer sequences were as follows: upstream primer (forward), 5' CCC AAT TCC TGG AGC
- 35 ATG AAG CTT CCA AA 3', and downstream primer (reverse complement), 5' GGG AAT TCA AGC GTA ATC CGG AAC ATC GTA TGG GTA GCT GCA GCC ACA GGC CTC TAC 3'. Plasmid from a cDNA

clone containing the entire  $\beta_E$  coding region was used as a source of template DNA, and PCR was performed essentially as described above. The PCR product (1.1-kbp) was digested with EcoRI, purified by agarose gel electrophoresis, and subcloned 5 into the pCDNA3 vector.

The activin  $\beta_A$  subunit was obtained by PCR, using an ovary cDNA library (Clontech) as a source of template DNA. Primer sequences were as follows: upstream primer (forward), 5' GCT GCC AGG ATG CCC TTG CTT 3', and downstream primer 10 (reverse complement), 5' CTA TGA GCA CCC ACA CTC 3'. PCR proceeded through 30 cycles of denaturation (94°C, 1.5 min), annealing (60°C, 1 min, 10 sec), and elongation (72°C, 1 min, 10 sec). The PCR product (1.3-kbp) was purified by agarose gel electrophoresis and then cloned into the TA cloning 15 vector (Invitrogen Corp., San Diego, CA). An expression plasmid coding for the mouse activin  $\beta_A$  subunit was then assembled into the pCDNA3 vector. An HA epitope was added to the 3' end of the coding sequence essentially as described above. The plasmid pbE #2 was used as a source of template 20 DNA, and PCR was performed essentially as described above. The PCR product was digested with EcoRI, purified by agarose gel electrophoresis, and subcloned into the pCDNA3 vector.

Expression Plasmids. To obtain homodimers of recombinant activin  $\beta_C$ ,  $\beta_E$ , and  $\beta_A$  subunits, transient 25 transfection was performed using standard protocols. Sambrook et al., 1989, supra. On the day of transfection, subconfluent 293T cells were fed fresh Dulbecco's Modified Eagles' Medium supplemented with 10% fetal calf serum (reagents from Gibco-BRL Life Technologies). Ten  $\mu$ g of 30 expression plasmid DNA were added to 0.5 ml of 0.25 M  $\text{CaCl}_2$  solution, and the mixture was added drop-wise to 0.5 ml Hepes-buffered saline (270 mM NaCl, 1.5 mM  $\text{NaPO}_4$ , 50 mM Hepes, pH 7.05). This mixture was kept at room temperature for 10 min and then added drop-wise to the medium of freshly fed 35 293T cells. After an overnight incubation at 37°C, the medium was replaced and cells were cultured for an additional 48 h. Medium samples were collected in the presence of

protease inhibitors (leupeptin, 1 mg/ml, aprotinin, 1 mg/ml, soybean trypsin inhibitor, 10 mg/ml, and pepstatin, 1 mg/ml; reagents from Sigma Chemical Co., St. Louis, MO), and stored at 4°C until use.

5

## 6.2 RESULTS AND DISCUSSION

A pair of degenerate PCR primers was designed based on conserved sequences in human bone morphogenetic protein-2 and -4 (VGWNDWI and VVEGCGC): upstream primer (forward), 5' GT(ACGT) GG(ACGT) TGG AA(CT) GA(CT) TGG AT 3'; and downstream primer (reverse complement), 5' CA (ACGT)CC (AG)CA (ACGT)CC (CT)TC (ACGT)AC (ACGT)AC 3'. Note that individual codons have been separated by a space and that degeneracy was achieved by adding a cocktail of nucleotides, as shown in parentheses, at selected wobble positions. PCR products were expected to be derived from within a single coding exon, and both cDNA and genomic DNA were used as templates. PCR was performed using a series of annealing temperatures until a band of the expected size of ~300-bp was obtained. The resultant PCR products were cloned into the TA cloning vector (Invitrogen) and then used to transform *E. coli*. Thousands of transformed *E. coli* colony forming units were available for screening, and several known TGF- $\beta$  superfamily members eventually were identified, including bone morphogenetic protein-2 and -4, activin  $\beta_A$ , activin  $\beta_B$ , and growth/differentiation factor-6 and -7 (not shown).

These efforts also led to the isolation of an apparently unique PCR fragment of 280 nucleotides that we designated DP-2. The DP-2 fragment was used as a probe to screen 500,000 plaque forming units from a mouse liver cDNA library. Three unique, overlapping cDNA clones that hybridized to DP-2 under high stringency conditions were purified from a pool of more than 20 positive isolates. DNA sequence analysis (all 3 clones) revealed an open reading frame of 1056 nucleotides within a 1837 nucleotide cDNA sequence (FIG. 1). The predicted sequence consisted of 352 amino acids with an estimated molecular weight (molw) of 39 kD and 4 potential N-

linked glycosylation sites. A Met codon (nucleotide 148) in a favorable context for translation initiation was provisionally designated the translational start site. Upstream of this ATG codon, we found four in-frame stop  
5 codons but no additional ATG codons. We also found a canonical TGF- $\beta$  CXCX sequence (Roberts, et al. 1990, The transforming growth factor betas. In: Sporn MB, Roberts AB (eds) Handbook of Experimental Pharmacology, 95 (Part I), Springer-Verlag, Heidelberg, pp. 419-472) preceding the stop  
10 translation signal (TAG). Based on sequence comparisons (described below), the 1056 nucleotide open reading frame sequence has provisionally been designated mouse activin  $\beta_c$ .

While cloning the mouse activin  $\beta_c$  genomic locus, we found a 300-bp BamHI genomic DNA fragment that was located  
15 downstream of the activin  $\beta_c$  gene and yet hybridized on a Southern blot under low stringency conditions with a  $\beta_c$  cDNA probe (data not shown). This BamHI fragment was subcloned into the plasmid vector pBluescript (Stratagene) and used as a probe to screen an additional 500,000 plaque forming units  
20 from the mouse liver cDNA library under high stringency conditions. Unique, overlapping cDNA clones, which represented a sequence of 2130-bp, were purified from a pool of more than 20 positive isolates. Sequence analysis (all 3 clones) revealed an open reading frame of 1050 nucleotides, a  
25 conceptual sequence of 350 amino acids, a predicted molecular weight of 39-kD, and 1 potential N-linked glycosylation site (FIG. 2B). A Met codon (nucleotide 216) in a favorable context for translation initiation was provisionally designated the translational start site. Upstream of this ATG  
30 codon, we found a single in-frame stop codon but no additional ATG codons. A canonical CXCX sequence preceding the stop translation signal (TAG) was also identified. As described below, a sequence comparison analysis suggests that the 1050 nucleotide open reading frame sequence codes for a  
35 novel activin  $\beta$  subunit, which has been designated mouse activin  $\beta_E$ .

The mouse activin  $\beta_c$  and  $\beta_e$  subunits are organized like other TGF- $\beta$  superfamily members (Roberts, et al., 1990, The transforming growth factor betas. In: Sporn MB, Roberts AB (eds) Handbook of Experimental Pharmacology, 95 (Part I), 5 Springer-Verlag, Heidelberg, pp. 419-472; Kingsley, D.M., 1994, Genes Develop 8:133-146). In both instances the initiator Met residue is followed by a characteristic signal peptide of ~20 amino acids. Downstream of the signal peptide both sequences appeared to be organized into propeptide and 10 mature growth factor domains, with putative endoproteolytic cleavage sites (i.e., clustered basic amino acids; see FIG. 1 and FIG. 2), at the junction of these domains. If these cleavage sites are in fact used, then the propeptide and mature growth factor domains of the mouse activin  $\beta_c$  subunit 15 will consist of approximately 216 and 116 amino acids, respectively, and the propeptide and mature growth factor domains of the mouse activin  $\beta_e$  subunit will consist of approximately 215 and 114 amino acids, respectively. Except for Mullerian inhibitory substance, all known members of the 20 TGF- $\beta$  superfamily have a cluster of basic residues approximately 120 amino acids from the C-terminus. At least in the case of the TGF- $\beta$ 's (Roberts et al., 1990, The transforming growth factor betas. In: Sporn MB, Roberts AB (eds) Handbook of Experimental Pharmacology, 95 (Part I), 25 Springer-Verlag, Heidelberg, pp. 419-472; Kingsley, D.M., 1994, Genes Develop 8:133-146), inhibins (Vale et al., 1990, The activins and inhibins. In: Sporn MB, Roberts AB (eds) Peptide Growth Factors and Their Receptors, 95 (Part II). Springer-Verlag, Heidelberg, pp. 211-248; Mason et al., 1985, 30 Nature 318:659-663), and bone morphogenetic protein-2 the mature form of the protein is known to be generated by endoproteolytic cleavage at these sites.

Several genomic clones were isolated under high stringency conditions in a screen of 1,000,000 plaque forming 35 units from a mouse genomic library using a  $\beta_c$  cDNA as the probe. Partial restriction and DNA sequence analyses of the overlapping inserts of these clones indicated that ~5-kbp of

mouse genomic DNA separates the 3' end of activin  $\beta_c$  and the 5' end of activin  $\beta_e$ , with the activin  $\beta_e$  locus downstream of activin  $\beta_c$  (FIG. 4). The organization of both genetic loci is similar, consisting of two coding exons separated by a single  
5 intervening sequence. Partial restriction analysis and partial DNA sequence analysis of overlapping genomic clones indicated that the size of the activin  $\beta_c$  intron is ~12-kbp. In contrast, DNA sequencing has shown that the size of the activin  $\beta_e$  intron is 234-bp. The 234-bp activin  $\beta_e$  intron  
10 sequence has been deposited in GenBank (accession number).

The predicted carboxy-terminal amino acid sequences of mouse activin  $\beta_c$  and  $\beta_e$  are highly homologous, with 61% amino acid identity over the mature growth factor domain. Both coding sequences contain a total of nine cysteine residues;  
15 spacing of the cysteines and the presence of other conserved amino acids is most characteristic of the activin subfamily and, to a lesser extent, TGF $\beta$ 1-5. In tabulating percent amino acid sequence identity within the mature growth factor domain, mouse activin  $\beta_c$  (the 1056 nucleotide coding sequence)  
20 showed 52% identity with human activin  $\beta_A$ , 50% identity with human activin  $\beta_B$ , 95% identity with human activin  $\beta_C$ , and 63% identity with *Xenopus* activin  $\beta_D$ . In comparison, mouse activin  $\beta_e$  (the 1050 nucleotide coding sequence) showed 45% identity with human activin  $\beta_A$ , 47% identity with human  
25 activin  $\beta_B$ , 64% identity with human activin  $\beta_C$ , and 62% identity with *Xenopus* activin  $\beta_D$ . In contrast, we found only 20-40% amino acid sequence identity when our mouse coding sequences were compared with other members of the TGF- $\beta$  superfamily. These data suggest that the 1056 nucleotide  
30 coding sequence most likely is the mouse homolog of human activin  $\beta_c$ . In contrast, the 1050 nucleotide coding sequence appears to be a novel activin subunit gene, which we have designated activin  $\beta_e$ . To our knowledge, this is the first report of the isolation of activin  $\beta_e$  from any species.

35 The relationships also suggest that activin  $\beta_A$  and  $\beta_B$  are most related to one another, while activin  $\beta_C$ ,  $\beta_D$ , and  $\beta_E$  represent a second subset of related sequences. Alignment of

the mature growth factor region of the five activin  $\beta$  subunits is shown in FIG. 3. We found that activin  $\beta_A$  and  $\beta_B$  share 63% amino acid identity, while activin  $\beta_C$ ,  $\beta_D$ , and  $\beta_E$  share 62% identity. The five activin  $\beta$  subunits share about 50% identity overall.

The pattern of mouse activin  $\beta_C$  and  $\beta_E$  gene expression was assessed by Northern analysis of several adult mouse tissues. In contrast to activin A and B, which are widely expressed in adult animals and humans (See, e.g., Meunier, H. et al., 1988, Proc. Natl. Acad. Sci. USA, 85:247-251), hybridization of a multiple tissue Northern blot with a specific probe from the 3' untranslated region of activin  $\beta_C$  identified a single major transcript of ~2.0-kb in the liver (FIG. 6). This band was identified with as little as 3 h exposure to x-ray film. Expression of the activin  $\beta_C$  gene by other tissues (heart, brain, spleen, lung, kidney, testis, and skeletal muscle) was not detected even if the blot was exposed to x-ray film for as long as 3 days (not shown). The activin  $\beta_E$  gene was also highly expressed in liver. Re-hybridization of the same multiple tissue Northern blot with as specific probe from the 3' untranslated region of activin  $\beta_E$  identified a single liver transcript of ~3.2-kb. This band was also identified with as little as 3 h exposure to x-ray film, and expression in other tissues was not detected even after exposure to x-ray film for up to 3 days (not shown).

The murine activin  $\beta_E$  clone was used as a probe to screen 500,000 plaque forming units from a human liver cDNA library under low stringency conditions. Two overlapping cDNA clones (representing a sequence of 1,764-bp) were purified from a pool of more than 20 positive isolates. Sequence analysis revealed a predicted open reading frame of 1050 nucleotides coding for a protein with an estimated molecular weight of 39 kD and 1 potential N-linked glycosylation site (Fig. 5). A MET initiator codon (beginning with nucleotide 77) is believed to be the site for translation initiation. A canonical CXCX sequence, preceding the stop translation signal (TAG), was also identified. A polyadenylation signal

has yet to be identified downstream of the stop codon. Sequence comparison analysis suggests that the 1050-bp open reading frame sequence codes for human activin  $\beta_E$ .

The human activin  $\beta_E$  subunit is organized like other TGF-  
5  $\beta$  superfamily members. The predicted initiator MET residue is followed by a characteristic signal peptide of ~20 amino acids. Downstream of the signal peptide, a predicted propeptide and mature growth factor domains have been identified. The domains are separated by a cluster of basic  
10 amino acids, creating one or more putative endoproteolytic cleavage sites. Cleavage at these sites will produce a propeptide and mature growth factor of ~215 and 114 amino acids, respectively.

The predicted carboxy-terminal amino acid sequences of  
15 mouse and human activin  $\beta_E$  are highly homologous, with >95% amino acid identity over the mature growth factor region. Both coding sequences contain a total of nine cysteine residues. The spacing of the cysteines and the presence of other conserved amino acids is most characteristic of the  
20 activins and to a lesser extent, TGF- $\beta$  itself. In tabulating percent amino acid sequence identity within the mature growth factor region, human activin  $\beta_E$  showed 45% identity with human activin  $\beta_A$ , 47% identity with human activin  $\beta_B$ , 64% identity with human activin  $\beta_C$ , and 62% identity with *Xenopus* activin  
25  $\beta_D$ . In contrast, only 20-40% amino acid sequence identity was found when human activin  $\beta_E$  was compared with other members of the TGF- $\beta$  superfamily.

This example reports the isolation and characterization of two murine and one human cDNA that code for members of the  
30 TGF- $\beta$  superfamily. The cDNAs, which are closely related, code for  $\beta$  subunits of activin. One of these subunits is likely to be the mouse homolog of human activin  $\beta_C$ , while the second and third are novel and have been designated the mouse and human activin  $\beta_E$  subunit. Taken together, the high degree  
35 of sequence identity between activin  $\beta_C$  and  $\beta_E$ , their clustered genomic organization, and their specific and strong expression pattern in adult liver suggest that these genes

evolved from a common ancestor that underwent a tandem duplication. These data also suggest that the activin  $\beta_c$  and  $\beta_e$  subunits may represent a unique subgroup of TGF- $\beta$ -like molecules.

- 5 To date, a total of five activin/inhibin  $\beta$  subunits and two inhibin  $\alpha$  subunits have been isolated and characterized. Whereas the activins were originally isolated from gonadal fluids by measuring the regulation of FSH release from primary rat anterior pituitary cells in vitro (Vale et al., 10 1986, Nature 321:776-779; Ling et al., 1986, Nature 321:779-782; Ling et al., 1986, Biochem Biophys Res Commun 138:1129-1137), activins have now been independently purified based on a number of different activities, including, but not limited to, erythroid differentiation (Yu et al., 1987, Nature 15 330:765-767; Eto et al., Biochem Biophys Res Commun 142:1095-1103), nerve cell survival (Schubert, D., et al., 1990, Nature 344:868-870), mesoderm induction in *Xenopus laevis* embryos (Slack J.M., 1994, Curr Biol 4:116-126), megakaryocyte differentiation (Fugimoto, K., 1991, Biochem. 20 Biophys. Res. Commun. 174:1163-1168), cardiac morphogenesis (Olson, E., et al., 1996, Science 272:671-676), bone growth (Luyten, F., et al., 1994, Exp. Cell Res. 210:224-229; Ogawa, Y., et al., 1992, J. Biol. Chem. 267:2325-2328), and somatostatin induction (Woodruff, T. et al., 1995, Annu. Rev. 25 Physiol. 57:219-244). Three activins (A, B, and AB) have been well characterized to date, each consisting of different combinations of  $\beta$  subunits ( $\beta_A\beta_A$ ,  $\beta_B\beta_B$ , and  $\beta_A\beta_B$ , respectively) that assemble into disulfide linked dimers. It is not yet clear if the activin  $\beta_c$ ,  $\beta_d$ , and  $\beta_e$  subunits assemble into 30 homodimers, heterodimers, or both. The recent isolation of these subunits suggests, however, that the number of combinatorial possibilities for activin may in fact be relatively large. On the other hand, inhibin was initially identified as a gonadal peptide that inhibited FSH release 35 from the anterior pituitary (Vale et al., 1990, The activins and inhibins. In: Sporn MB, Roberts AB (eds) Peptide Growth Factors and Their Receptors, 95 (Part II). Springer-Verlag,

Heldelberg, pp. 211-248; Mason et al., 1985, Nature 318:659-663; Rivier, J., et al., 1985, Biochem. Biophys. Res. Commun. 133:120-127). The major gonadal sites of inhibin synthesis are Sertoli and granulosa cells. Inhibin A and B are also  
5 expressed in the placenta, and in the adult spleen and nervous system. In mice of both genders, inhibin appears to be an important negative regulator of gonadal stromal cell proliferation-i.e., inhibin-deficient mice develop mixed or incompletely differentiated gonadal stromal tumors that  
10 consist of granulosa cells (female mice) and Sertoli cells (male mice) (Matzuk, M., et al., 1992, Nature 360:313-319).

7. **EXAMPLE: DIRECT TRANSFER AND EXPRESSION OF  
ACTIVIN  $\beta_c$  EXPRESSION PLASMID DNA  
CAUSES NEW BONE FORMATION IN VIVO**

15 Direct in vivo gene transfer was used to determine if activin  $\beta_c$  homodimers possess osteoinductive activity. Degradable matrices containing plasmid DNA (gene-activated matrices, or GAMs) were implanted into segmental gaps in the adult rat femur. Implantation of a GAM containing activin  $\beta_c$   
20 expression plasmid DNA resulted in the biological response of new bone filling the gap. This experiment demonstrates that overexpression of activin  $\beta_c$  causes new bone to form in vivo.

7.1 **MATERIALS AND METHODS**

25 **Animal model.** To create a 5 mm osteotomy, four 1.2 mm diameter pins were screwed into the femoral diaphysis of normal adult Sprague-Dawley rats under general anesthesia and with constant irrigation. A surgical template guided parallel pin placement, which was confirmed by fluorography  
30 (pins were set 3.5 mm from the edge of the fixator plate and 2.5 mm apart). An external fixator plate (30 x 10 x 5 mm) was then secured on the pins. External fixator plates were fabricated with aluminum alloy on a CNC mill to ensure high tolerances. Prefabricated fasteners with associated  
35 lockwashers and threaded pins were made of stainless steel. All fixator parts were sterilized with ethylene oxide gas

prior to surgery. Five mm segmental defects were created at mid-shaft with a Hall Micro 100 oscillating saw (Zimmer Inc., Warsaw, IN). Collagen sponges were held in the osteotomy gap until surrounded by clotted blood; preliminary studies showed  
5 that this maneuver fixed the sponge within the osteotomy site. The skin incision was closed with staples. The fixator provided the necessary stability so that animal ambulation was unlimited.

10     Preparation of Plasmid DNA. An established bacterial cell strain that contains an activin  $\beta_c$  expression plasmid was grown in liquid culture according to established protocols. Plasmids were purified in an endotoxin-free manner using reagents and protocols from Qiagen Inc.

15     Preparation of Gene-Activated Collagen Sponges. To 1.5 ml of a PLGA/chloroform solution (3% w/v), 50/50 polylactic polyglycolic acid co-polymer, average MW 90,000, inherent viscosity 1.07) was added 0.2 ml of a solution containing  
20 expression plasmid DNA encoding activin  $\beta_c$  (1 mg DNA, 10 mM Tris, 0.1 mM EDTA, pH 7.4). The solution was emulsified by vortexing for 2 minutes followed by sonicating for 30 seconds at about 0°C using a microtip probe-type sonicator at 55 Watts output.

25     Radiography. Weekly plain film radiographs (posterior-anterior view) were obtained while animals were awake using a portable X-ray unit (GE, model 100).

30     Histology. Tissues were prepared for light microscopy using standard methods of tissue fixation, demineralization, paraffin embedding, sectioning, and staining.

## 35                                   7.2   RESULTS

Osteotomy Model. Whereas osteotomy repair in the rat is completed by 9 weeks post-surgery, the manner of repair

depends on the size of the gap: a 2 mm gap heals by bony union, but a 5 mm gap heals by fibrous nonunion. Control animals maintained for up to 13 weeks post-surgery confirmed the observation that 5 mm gaps typically heal by fibrous nonunion. Weekly plain film radiography and histology demonstrated that bone did not form in animals that received either a 5 mm osteotomy alone (n=3), a 5 mm osteotomy plus a PLGA matrix (n=10), or a 5 mm osteotomy plus a PLGA matrix containing plasmid DNA (n=23). All 36 control gaps healed by deposition of fibrous tissue. Control femurs exhibited focal periosteal new bone formation (a complication of pin placement). A focal, transient inflammatory response (lymphocytes and macrophages) in gap tissues was also observed post-surgery.

15

**Activin  $\beta_c$  Gene Transfer.** A full length mouse activin  $\beta_c$  cDNA was isolated by library screening and subcloned into the pCDNA3 eukaryotic expression vector (Invitrogen). To specifically detect recombinant proteins, the 3' end of the activin  $\beta_c$  coding sequence was modified by addition of a hemagglutinin (HA) epitope. Recombinant activin  $\beta_c$  was expressed from this construct (pGAM3) using an *in vitro* transcription and translation protocol. Immunoprecipitation studies also established the ability of the HA epitope to be recognized by an anti-HA polyclonal antibody.

Biosynthesis of recombinant  $\beta_c$  was evaluated following transient transfection of cultured 293T cells with pGAM3 plasmid DNA: as demonstrated by immunoprecipitation, activin  $\beta_c$  molecules were assembled into homodimers, secreted, and processed as expected. FIG. 6.

PLGA matrices containing pGAM3 DNA were placed in the gap of three adult rats maintained for 4-24 weeks. Microscopic foci of new bone, originating from both surgical margins, were observed as early as 4 weeks after surgery. Consistent with a classic description of bone formation by autoinduction, these foci consisted of bony plates surfaced by large cuboidal osteoblasts and supported by a cellular

connective tissue composed of pleomorphic spindled fibroblasts and capillary vessels. The radiographic appearance of the contralateral (unoperated) femur was unchanged in all three cases (not shown), implying that the effects of gene transfer and activin  $\beta_c$  overexpression were limited to the osteotomy gap. These data demonstrate that activin  $\beta_c$  homodimers possess osteoinductive activity.

**8. EXAMPLE: RECOMBINANT ACTIVIN  $\beta_c$  HOMODIMERS STIMULATE HEMATOPOIESIS IN VIVO**

Human bone marrow biopsies were cultured in the presence of media conditioned with recombinant activin  $\beta_c$  to determine the effect, if any, of activin  $\beta_c$  homodimers on hematopoiesis. The experiment demonstrates that activin  $\beta_c$  homodimers are capable of stimulating hematopoiesis.

**8.1 MATERIALS AND METHODS**

Production of Activin  $\beta_c$  Conditioned Media. Transient transfection was performed as described using pGAM3 plasmid DNA, 293T cells, and standard protocols. On the day of transfection, sub-confluent 293T cells were fed fresh Dulbecco's Modified Eagles's Medium supplemented with 10% fetal calf serum (reagents from Gibco-BRL Life Technologies, Inc.). Ten  $\mu$ g of Plasmid DNA were added to 0.5 ml of 0.25 M  $\text{CaCl}_2$  solution, and the mixture was added drop-wise to 0.5 ml Hepes-buffered saline (270 mM NaCl, 1.5 mM  $\text{NaPO}_4$ , 50 mM Hepes, pH 7.05). This mixture was kept at room temperature for 10 min and then added drop-wise to the medium of freshly fed 293T cells. After an overnight incubation at 37°C, the medium was replaced and cells were cultured for an additional 48 h. For metabolic labeling studies, transfected cells were re-fed with Dulbecco's Modified Eagle's Medium lacking methionine and cysteine and supplemented with 5% dialyzed fetal calf serum (all reagents from Gibco-BRL Life Technologies, Inc.) and 200 mCi [ $^{35}\text{S}$ ] Met and [ $^{35}\text{S}$ ] Cys (Translabel Cys and Met, ICN). Cells were incubated for an additional 24 h at 37°C, medium samples were collected in the

presence of protease inhibitors (Leupeptin, 1 mg/ml, Aprotinin, 1 mg/ml, Soybean trypsin inhibitor, 10 mg/ml, and Pepstatin, 1 mg/ml; Sigma), and stored at 4°C until use.

5        **Human Bone Marrow Cultures.**    Human bone marrow aspirates were obtained from normal volunteers after informed consent. Long-term bone marrow cultures were established exactly as described by Gartner and Kaplan (1980, Proc. Natl. Acad. Sci. USA, 4756-4759). For immunolocalization, two to  
10 three coverslips were placed in each culture and removed weekly for immunocytochemistry. Determination of hematopoietic progenitor cell frequency and the total number of hematopoietic progenitor cells was performed as described previously (Long et al., 1990, J. Clin. Invest. 86:1387-  
15 1395). Assays were performed in triplicate.

## 8.2 RESULTS

### **Activin $\beta_c$ Conditioned Media Stimulates Hematopoiesis.**

Strong trends or statistically significant stimulation of  
20 erythropoiesis, granulopoiesis, and megakaryopoiesis were observed when activin  $\beta_c$  conditioned media was added to human marrow aspirate cultures (FIG. 9). In addition, the activin  $\beta_c$  conditioned media caused a two-fold increase in the total number of hematopoietic progenitor cells. These data  
25 demonstrate that activin  $\beta_c$  homodimers are capable of stimulating hematopoiesis.

### 9. EXAMPLE:    **ACTIVIN $\beta_c$ HOMODIMERS BIND LATENT TGF- $\beta$ BINDING PROTEIN-3**

30        The latent TGF- $\beta$  binding protein (LTBP) is a component of the large latent TGF- $\beta$  complex produced by platelets and other cells. Genes coding for three LTBPs have been isolated to date; two gene products (LTBP-1 and LTBP-2) have been shown to bind latent TGF- $\beta$ 1 via a disulfide bond, whereas  
35 LTBP-3 was isolated only recently and its binding potential has yet to be determined. To address this question, we transiently co-transfected cultured cells with expression

plasmids coding for mouse LTBP-3 and human TGF- $\beta$ 1, and studied complex formation using immunoprecipitation, SDS-PAGE and autoradiography (Yin et al., submitted). The results indicated that LTBP-3 also covalently binds latent TGF- $\beta$ 1 during assembly of the latent complex. The co-transfection strategy was also used to show for the first time that the site of disulfide bond formation is the 8-cysteine TGF-bp structural motif in LTBP-3). These results were both confirmed and extended by additional co-transfection studies that employed TGF-bp expression plasmids from mouse LTBP-2. Together, these studies identify a new functional role for the TGF-bp motif during assembly of latent TGF- $\beta$ 1 complexes. The results have important implications for TGF- $\beta$  biosynthesis, storage, and targeting.

LTBP-3 has a total of three TGF-bp structural motifs, only two of which bind TGF- $\beta$ 1 with affinity. We therefore sought to determine if the third structural motif was able to bind related molecule-i.e., other members of the TGF- $\beta$  superfamily. In particular, we sought to determine if TGF- $\beta$ -like molecules with propeptides that have a conserved pattern of cysteine residue number and spacing would also bind LTBP-3. To address this question, a similar experimental design was employed: we transiently co-transfected cultured cells with expression plasmids coding for mouse LTBP-3 and activin  $\beta_c$ , and studied complex formation using immunoprecipitation, SDS-PAGE and autoradiography. The experiment demonstrates that the third 8-cysteine TGF-bp structural motif of LTBP-3 covalently binds activin  $\beta_c$ . The results demonstrate for the first time that LTBP-3 is a binding protein for activin PC homodimers, and they extend the functional role for the TGF-bp motif during activin  $\beta_c$  homodimer assembly.

### 9.1 MATERIALS AND METHODS

Assembly of pLTBP-3 fl. Yin et al. (1995) assembled and characterized a mammalian expression vector that codes for mouse LTBP-3 (pLTBP-3 fl; see Table 1, #1). Following transient transfection, this study showed that mouse LTBP-3

is precipitated by polyclonal antiserum #274, which recognizes a 12 amino acid epitope (GESVASKHAIYA) within the upstream, proline- and glycine-rich sequence of mouse LTBP-3.

- 5        Assembly of pLTBP-3/pept. Yin et al. assembled and characterized the pLTBP-3/pept expression vector. To this end, pLTBP-3 fl was digested with EcoRI and HindIII to yield two DNA fragments that were cleanly resolved by agarose gel electrophoresis. One fragment, consisting of an unwanted
- 10 portion of LTBP-3 coding sequence, was discarded. The second fragment—which included the intact plasmid backbone, CMV promoter, stop translation signal, and polyadenylation element—was designated pLTBP-3/pept (see Table 1, #2). The HindIII site is located 646 bp downstream of the Ltbp-3
- 15 initiator Met codon. Therefore, pLTBP-3/pept codes for an amino-terminal fragment of mouse LTBP-3 that includes the normal Kozak consensus and signal peptide; a short amino-terminal sequence unique to LTBP-3; two EGF-like repeats; and a portion of the upstream proline- and glycine-rich domain.
- 20 The latter includes the antibody #274 epitope (GESVASKHAIYA). As described below, the pLTBP-3/pept expression vector functioned in 293T cells in essentially the same way as pLTBP-3 fl—i.e., pLTBP-3/pept was used to generate a series of defined LTBP-3 peptide fragments whose potential to bind
- 25 TGF- $\beta$ 1 could be directly tested by immunoprecipitation with antibody #274.

- Assembly of pLTBP-3/TGF-bpA. pLTBP-3/pept facilitated the assembly of a plasmid encoding a defined fragment of
- 30 mouse LTBP-3. The following is a detailed description of how this plasmid, pLTBP-3/TGF-bpA, was assembled (see Table 1, #3; also see Fig. 1). PCR was used to generate a cDNA fragment that coded for the TGF-bpA structural motif from LTBP-3. The upstream PCR oligonucleotide primer contained a
- 35 NheI site in frame with 6/12 codons of the 274 peptide (KHAIYA) plus the 5' portion of the cDNA sequence that coded for the EGF-like repeats (the sequence was obtained from

GenBank™/EMBL Data Bank accession L40459). The downstream PCR primer contained an XhoI site plus the 3' portion of the TGF-bpA cDNA sequence. The PCR template consisted of pLTBP-3 fl plasmid DNA, and PCR was conducted as described (Chen et al., 1993). Following amplification, the PCR fragment was digested with NheI and XhoI, purified by agarose gel electrophoresis, and ligated with the pLTBP-3/pept expression vector after it had been digested with the same restriction enzymes and purified. Digestion with XhoI preserved the stop translation and polyadenylation elements within the vector. Competent bacteria were transformed by the ligation mixture, and colony forming units were isolated and used to prepare plasmid DNA. To insure its integrity, pLTBP-3/TGF-bpA was characterized by DNA sequencing.

15     Assembly of pGAM3 and transfection were performed as described above.

Immunoprecipitation. For immunoprecipitation, 10 ml (neat) of antibody was added to 1-5 ml of radiolabelled medium proteins. The mixture was incubated with shaking at room temperature for 2 h. Protein A-Sepharose CL-4B beads were added (200 ml, 10% suspension), and this mixture was incubated with shaking for 2 h at room temperature. Immunoprecipitated proteins were pelleted by brief centrifugation, and the pellet was washed 4 times with PBS-TD buffer (0.38 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1% Triton X-100, 0.5% deoxycholate) and 3 times with PBS. Protein loading dye (2x) was then added, with or without reducing agent (10% 2-mercaptoethanol), and the samples were boiled for 5 min. Samples were then fractionated using 4-18% gradient SDS-polyacrylamide gel electrophoresis (Yin et al., 1995). Cold molecular weight markers (200-14.3 kDa, Rainbow mix, Amersham Corp.) were used to estimate molecular weight. The gel was dried and exposed to film for the indicated time at room temperature.

35

## 9.2 RESULTS

293T mammalian cells were programmed by transient co-transfection with pLTBP-3/TGF-bpA and pGAM3, and then radiolabelled with [<sup>35</sup>S] Cys and [<sup>35</sup>S] Met for 16 h. Medium proteins were harvested, and immunoprecipitation studies were carried out.

Neither LTBP-3 nor activin  $\beta_c$  were identified in conditioned medium of untransfected 293T cells that were radiolabelled and immunoprecipitated with the anti-LTBP-3 and anti-HA antibodies. Following immunoprecipitation with the anti-HA antibody, conditioned medium of cells transfected with pGAM3 alone contained the activin propeptide and mature growth factor but not the LTBP-3 TGF-bpA repeat. Under reducing conditions, the propeptide monomer migrated as a single band of ~40 kDa, and mature activin  $\beta_c$  migrated as a single band of ~14 kDa. Following immunoprecipitation with the anti-HA antibody, the conditioned medium of 293T cells transfected with pLTBP3/TGF-bpA alone contained the bpA motif (a peptide of ~30 kDa) but not the latent form of activin  $\beta_c$ . A final set of controls was based on immunoprecipitation of conditioned media with the 'wrong antibody': activin  $\beta_c$  could not be identified if conditioned medium was immunoprecipitated with the anti-LTBP-3 antibody, and, conversely, the bpA motif could not be identified if the medium sample from conditioned medium of 293T cells transfected with pLTBP3/TGF-bpA was immunoprecipitated with the anti-HA antiserum. These control data have been obtained independently three times, attesting to the reproducibility of the transient co-transfection system.

Co-transfection experiments provided evidence of a specific interaction between the latent form of activin  $\beta_c$  and the TGF bpA structural motif of LTBP-3. For both the anti-LTBP-3 and anti-HA immunoprecipitates, the bpA motif again migrated as a band of ~30 kDa, the activin  $\beta_c$  propeptide migrated as a dispersed band of ~40 kDa, and mature activin  $\beta_c$  migrated as a single band of ~14 kDa.

Therefore, the data establishes that LTBP-3 binds activin  $\beta_c$  homodimers via a covalent reducible crosslink -i.e., a disulfide bond that is stable to boiling in SDS. Moreover, co-immunoprecipitation by both anti-LTBP-3 and 5 anti-HA antibodies provides strong evidence that the binding interaction between LTBP-3 and activin  $\beta_c$  was specific and, therefore, not due to an artifact of the transient co-transfection system.

**10**                      10. DEPOSIT OF MICROORGANISMS

The following organisms were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852.

<u>15 Strain Designation</u>	<u>Containing</u>	<u>Accession No.</u>
pbHE	human activin $\beta_E$	
pbC	murine activin $\beta_C$	
pbE	murine activin $\beta_E$	

The present invention is not to be limited in scope by  
20 the exemplified embodiments or deposited organisms which are  
intended as illustrations of single aspects of the invention,  
and any clones, DNA or amino acid sequences which are  
functionally equivalent are within the scope of the  
invention. Indeed, various modifications of the invention in  
25 addition to those described herein will become apparent to  
those skilled in the art from the foregoing description and  
accompanying drawings. Such modifications are intended to  
fall within the scope of the appended claims.

It is also to be understood that all base pair sizes  
30 given for nucleotides are approximate and are used for  
purposes of description.

<b>MICROORGANISMS</b>	
Optional Sheet in connection with the microorganism referred to on page <u>112</u> , lines <u>10-35</u> of the description *	
<b>A. IDENTIFICATION OF DEPOSIT *</b>	
Further deposits are identified on an additional sheet *	
Name of depositary institution *	
American Type Culture Collection	
Address of depositary institution (including postal code and country) *	
12301 Parklawn Drive Rockville, MD 20852 US	
Date of deposit * <u>November 20, 1996</u> Accession Number * <u>97804</u>	
<b>B. ADDITIONAL INDICATIONS *</b> (leave blank if not applicable). This information is continued on a separate attached sheet	
<b>C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *</b> (If the indications are not all designated States)	
<b>D. SEPARATE FURNISHING OF INDICATIONS *</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office)	
_____ (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau *	
was	
_____ (Authorized Officer)	

Form PCT/RO/134 (January 1981)

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive  
Rockville, MD 20852  
US

Accession No.

Date of Deposit

97805

November 20, 1996

97806

November 20, 1996

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule containing the nucleotide sequence of liver activin  $\beta_E$  gene.
- 5 2. An isolated nucleic acid molecule which encodes a liver activin  $\beta_E$ , or a fragment thereof, having a nucleotide sequence that:
  - 10 (a) encodes the amino acid sequence shown in FIG. 2 or the amino acid sequence encoded by the cDNA contained in cDNA clone \_\_\_\_\_ as deposited with the ATCC having accession No. \_\_\_\_\_; or
  - (b) hybridizes under stringent conditions to the nucleotide sequence of (a) or to its complement.
- 15 3. An isolated nucleotide sequence encoding a liver activin polypeptide corresponding to the N-terminal signal peptide, propeptide, or mature growth factor domain or the mature growth factor domain is deleted.
- 20 4. An isolated nucleotide sequence encoding a chimeric protein comprising the polypeptide of Claim 3 fused to a heterologous polypeptide.
- 25 5. A nucleotide vector containing the nucleotide sequence of Claim 1, 2, 3, or 4.
6. An expression vector containing the nucleotide sequence of Claim 1, 2, 3, or 4 in operative association with  
30 a nucleotide regulatory sequence that controls expression of the nucleotide sequence in a host cell.
7. An expression vector containing the nucleotide sequence shown in FIG. 1, or encoding the amino acid sequence  
35 shown in FIG. 1 or the amino acid sequence encoded by the cDNA contained in cDNA clone \_\_\_\_\_ as deposited with the ATCC having accession No. \_\_\_\_\_; in operative association

with a nucleotide regulatory sequence that controls expression of the nucleotide sequence in a host cell.

8. The expression vector of Claim 6, in which said  
5 regulatory sequence is selected from the group consisting of the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage  $\lambda$ , the control regions of fd  
10 coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast  $\alpha$ -mating factors.

9. The expression vector of Claim 7, in which said  
15 regulatory sequence is selected from the group consisting of the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage  $\lambda$ , the control regions of fd  
20 coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast  $\alpha$ -mating factors.

10. A genetically engineered host cell that contains  
25 the nucleotide sequence of Claim 1, 2, 3, or 4.

11. A genetically engineered host cell that contains the nucleotide sequence of Claim 1, 2, 3, or 4 in operative association with a nucleotide regulatory sequence that  
30 controls expression of the nucleotide sequence in the host cell.

12. The genetically engineered host cell of Claim 11 in which the host cell is a hepatocyte.

35

13. A genetically engineered host cell that contains the expression vector of Claim 7.

14. The genetically engineered host cell of Claim 13 in which the host cell is a hepatocyte.

15. An isolated liver activin protein.

5

16. An isolated liver activin protein having the amino acid sequence shown in FIG. 1 or 2, or the amino acid sequence encoded by the cDNA contained in cDNA clone \_\_\_\_\_ as deposited with the ATCC having accession No. \_\_\_\_\_, or the  
10 amino acid sequence encoded by the cDNA contained in cDNA clone \_\_\_\_\_ as deposited with the ATCC having accession No. \_\_\_\_\_.

17. A polypeptide having an amino acid sequence  
15 corresponding to the N-terminal signal peptide, propeptide, mature growth factor domain of liver activin protein, or a deletion mutant of the liver activin protein in which the N-terminal signal peptide, propeptide, or the mature growth factor domain is deleted.

20

18. A chimeric protein comprising the polypeptide of Claim 17 fused to a heterologous polypeptide.

19. An antibody that immunospecifically binds the liver  
25 activin protein of Claim 16 or 17.

20. An antibody that immunospecifically binds the polypeptide of Claim 17.

30 21. A method for diagnosing cell growth or differentiation disorders in a vertebrate comprising measuring liver activin gene expression in a patient sample.

22. The method of Claim 21 in which expression is  
35 measured by detecting mRNA transcripts of the liver activin gene.

23. The method of Claim 21 in which expression is measured by detecting the liver activin gene product.

24. A method for diagnosing cell growth or  
5 differentiation disorders in a vertebrate, comprising detecting a liver activin gene mutation contained in the genome of the vertebrate.

25. A method for regulating cell growth or  
10 differentiation in a vertebrate, comprising administering liver activin compound to the vertebrate in an amount sufficient to stimulate cell growth or differentiation.

26. The method of Claim 25 in which the compound is a  
15 polypeptide corresponding to the mature growth factor domain of the liver activin or a portion of the mature growth factor domain that binds liver activin receptor, a deletion mutant liver activin protein lacking the endoproteolytic motif, propeptide or mature growth factor domain, or a chimeric  
20 fusion protein comprising the mature growth factor domain of the liver activin, or a portion of the mature growth factor domain that binds liver activin receptor deletion mutant fused to a heterologous polypeptide.

25 27. The method of Claim 25 in which the compound is an antagonist that binds to the liver activin receptor and inhibits activation of the receptor.

28. The method of Claim 25 in which the compound binds  
30 to endogenous liver activin and neutralizes liver activin activity.

29. The method of Claim 28 in which the compound is delivered to the vertebrate by administering a genetically  
35 engineered host cell that expresses and secretes the polypeptide or fusion protein in the vertebrate.

30. The method of Claim 28 in which the compound is an anti-idiotypic antibody, or an Fab portion thereof, that mimics the mature growth factor domain of the liver activin and neutralizes endogenous liver activin receptor.

5

31. A method for regulating cell growth or differentiation in a vertebrate, comprising administering a compound to the mammal in an amount sufficient to inhibit expression of the liver activin in vivo.

10

32. The method of Claim 31 in which the compound is delivered to the liver.

33. The method of Claim 31 in which the compound is an antisense oligonucleotide that inhibits translation of mRNA transcripts that encode the liver activin.

34. The method of Claim 31 in which the compound is a ribozyme that inhibits translation of mRNA transcripts that encode the liver activin.

35. The method of Claim 31 in which the compound is an oligonucleotide that forms a triple helix with the regulatory region of the liver activin gene and inhibits transcription.

25

36. The method of Claim 31 in which the compound is a recombinant DNA construct that inactivates the liver activin gene or its regulatory region via targeted homologous recombination.

30

37. A method for regulating cell growth or differentiation in a vertebrate, comprising administering a compound to the vertebrate in an amount sufficient to inhibit signal transduction induced by binding of liver activin to an activin receptor.

35

38. A method for treating liver disorder, comprising administering an effective amount of liver activin gene product antagonist to a patient in need of such therapy.

5        39. A method for regulating cell growth or differentiation, comprising administering a compound to a vertebrate in an amount sufficient to up regulate expression of a liver activin in the vertebrate.

10       40. The method according to Claim 42 in which the compound is delivered to the liver.

41. The method of Claim 42 in which the vertebrate expresses a defective liver activin and the compound  
15 comprises a nucleotide construct encoding a functional liver activin controlled by a regulatory region that directs expression of the functional liver activin in target cells in the vertebrate.

20       42. The method of Claim 42 in which the vertebrate expresses a mutant liver activin and the compound comprises a nucleotide construct encoding a wild-type liver activin that corrects the endogenous mutation via targeted homologous recombination.

25       43. A method of enhancing the growth or regeneration of liver tissue comprising treating the liver tissue with an effective amount of liver activin gene product agonist.

30       44. The method of Claim 39 in which the liver tissue is extracorporeal.

45. The method of Claim 39 in which the liver tissue is intracorporeal.

35

46. A method for inducing bone growth in a vertebrate comprising administering an osteogenically effective amount of liver activin compound to the vertebrate.

5        47. The method of Claim 46 in which the compound comprises a polypeptide corresponding to the mature growth factor domain of the liver activin or a portion of the mature growth factor domain that binds liver activin receptor, a deletion mutant liver activin protein lacking the  
10 endoproteolytic motif, propeptide or mature growth factor domain, or a chimeric fusion protein comprising the mature growth factor domain of the liver activin, or a portion of the mature growth factor domain that binds liver activin receptor deletion mutant fused to a heterologous polypeptide.

15

48. The method of Claim 46 further comprising a compound selected from the group consisting of a TGF- $\beta$ , a BMP, and bone marrow.

20        49. The method of Claim 46, wherein the bone growth is systemic.

50. The method of Claim 46, wherein the bone growth is local.

25

51. A method for stimulating hematopoiesis in a vertebrate comprising administering a hematopoietically effective amount of liver activin compound to the vertebrate.

30        52. The method of Claim 51 in which the compound comprises a polypeptide corresponding to the mature growth factor domain of the liver activin or a portion of the mature growth factor domain that binds liver activin receptor, a deletion mutant liver activin protein lacking the  
35 endoproteolytic motif, propeptide or mature growth factor domain, or a chimeric fusion protein comprising the mature growth factor domain of the liver activin, or a portion of

the mature growth factor domain that binds liver activin receptor deletion mutant fused to a heterologous polypeptide.

53. The method of Claim 51 further comprising a  
5 compound selected from the group consisting of an iron  
preparation, vitamin B12, folic acid, an adrenocortical  
steroid, an erythropoietin, a testosterone, a progenitor cell  
stimulator, insulin-like growth factor, a prostaglandin,  
serotonin, cyclic AMP, prolactin, triiodothyronine,  
10 methenolene, stanozolol, nandrolone, and androgen and a  
erythrocyte.

54. The method of Claim 51 wherein the compound  
contains erythropoietin.

15

55. The method of Claim 51 wherein the compound  
stimulates erythropoiesis.

20

25

30

35

G CTG TAT CTC TGA CAA AAA GGA GTC ATG CCA GTC GGA GGT CAG TCA CAT TCC TCC 55  
 CAG GGT CCC TGG TGC CCA GGA CAG AGT TGA AGC CAC TCC CGT TGA GAC CCT GAA TAT 112  
 AGG CTT TGG GTC CTT TAA GGA GGC TAT CCT CCA GCA ATG GCC TCC TCC TTG CTC CTG 169  
 M A S S L L L 7  
 GCT CTT CTG TTC CTG ACT CCA ACC ACA GTA GTG AAC CCC AAA ACT GAG GGT CCA TGC  
 A L L F L T P T T V V N P K T E G P C 26  
 CCA GCA TGT TGG GGT GCC ATC TTT GAC CTG GAG AGC CAG CGG GAG CTG CTT CTC GAT  
 P A C W G A I F D L E S Q R E L L L D 45  
 TTG GCC AAG AAA AGT ATC CTG GAC AAG CTG CAC CTC AGC CAG CGC CCC ATA CTC AGT  
 L A K K S I L D K L H L S Q R P I L S 64  
 CGG CCA GTG TCC AGA GGG GCT CTC AAG ACC GCG CTG CAG CGC CTC CGC GGG CCT CGA  
 R P V S R G A L K T A L Q R L R G P R 83  
 CGG GAA ACC CTG TTG GAG CAT GAC CAG AGA CAA GAA GAA TAT GAG ATC ATC AGC TTT  
 R E T L L E H D Q R Q E E Y E I I S F 102  
 GCT GAC ACA GAC CTC TCC AGC ATC AAC CAG ACC CGG CTC GAG TTC CAC TTC TCT GGT  
 A D T D L S S I N Q T R L E F H F S G 121  
 AGA ATG GCC AGT GGC ATG GAG GTC CGG CAG ACC CGC TTC ATG TTC TTC GTG CAG TTC  
 R M A S G M E V R Q T R F M F F V Q F 140  
 CCC CAC AAT GCC ACC CAG ACC ATG AAT ATA AGA GTT CTT GTG CTA AGA CCA TAT GAC  
 P H N A T Q T M N I R V L V L R P Y D 159  
 ACC AAC CTC ACC TTG ACA AGT CAG TAC GTG GTG CAG GTG AAT GCC AGT GGC TGG TAC  
 T N L T L T S Q Y V V Q V N A S G W Y 178  
 CAG CTT CTC CTG GGA CCT GAA GCT CAA GCT GCT TGC AGC CAG GGA CAC CTT ACT CTG  
 Q L L L G P E A Q A A C S Q G H L T L 197  
 GAG CTG GTA CCA GAA AGC CAG GTG GCC CAC AGT TCC TTG ATC CTG GGC TGG TTT TCC  
 E L V P E S Q V A H S S L I L G W F S 216  
 CAC AGG CCT TTT GTG GCA GCC CAG GTA AGG GTT GAG GGC AAG CAT CGG GTT CGC CGG  
 H R P F V A A Q V R V E G K H R V R R 235  
 CGA GGT ATC GAT TGC CAG GGG GCG TCC AGG ATG TGC TGT CGA CAA GAG TTC TTC GTA  
 R G I D C Q G A S R M C C R Q E F F V 254  
 GAC TTC CGT GAG ATT GGC TGG AAT GAC TGG ATC ATC CAG CCT GAA GGC TAT GCC ATG  
 D F R E I G W N D W I I Q P E G Y A M 273  
 AAC TTC TGC ACC GGG CAG TGC CCA CTA CAT GTG GCA GGC ATG CCT GGC ATC TCT GCC  
 N F C T G Q C P L H V A G M P G I S A 292  
 TCC TTT CAC ACT GCA GTG CTG AAT CTG CTC AAA GCC AAC GCA GCT GCT GGC ACC ACT  
 S F H T A V L N L L K A N A A A G T T 311  
 GGC AGG GGC TCG TGC TGC GTG CCT ACA TCT CGG CGC CCT CTG TCT TTG CTC TAC TAT  
 G R G S C C V P T S R R P L S L L Y Y 330  
 GAC AGG GAC AGC AAC ATT GTC AAG ACG GAT ATA CCT GAC ATG GTG GTC GAG GCC TGC  
 D R D S N I V K T D I P D M V V E A C 349  
 GGG TGT AGT TAG CTT ATG GGT GAT ACA GGC TGC CTG AGG TAG AAT GGC CTT CCT CAG  
 G C S 352  
 GAA GGG GAA ACT CTG TTC CCA CTT CTG TCC AGA ATG GAA ACA CCT TTC TAA GCA TGC  
 AGA CAT CCC TCT GTG GAC TTC AGG GGA TCC ACC TCT AAA GAG AGT CAC TAG TGA CCA  
 ACA GCC TTT CTC TCT CCT GGG ACA TGG TTG ACC CAG TAC ACC CAT CCT CAG CCT TAA  
 GTT AGA GGC TAA TCG ACT GCC TAC CAC AAG CAA TGT CAT TTT GTT CCT AGC AAA CAC  
 ACC CTT AGC TCT CCC TTA GTC AAC TAT GTA ATC TAC TCT GCC TCC CTG ACC CTG CCA  
 CCG GAA GGT TCC TAT TCC ACG ATG ATA TGC CTT AGT GTC TCC CCT TGA ATT CTG TGG  
 CTC TCC GAA GAA CCC CTT CAT CAG GGT CAC TGA AGA TTA TAT TGC TGC CTT CCA AAG  
 AAA GGC TCA TCT CTC AAA TCT GGA GAC CCG AGC CCA GAT GTG CTA CTA AAT AGT GTT  
 CTC TGC CCT CTT CTG CCC TCG GGG GTG ACA TAC CAA GTC TCA TTG GTT GGA CAT TCC  
 TCT AGT TTC CAG CTG AAT TTG ACC AAA AGG AGA CTC CAG TTG GAA ACT AAA GCC TAG  
 GAT AAC ACA GAA AAA

FIG. 1

350

FIG. 2

	C	CC	C	C
Activin $\beta_A$ (h)	CDGKV----	NICCKKQFFVSF-KDIGWNDWIIAPSGYHANYCEGECPSHIAGTSGSSLSE		
Activin $\beta_B$ (h)	COGRT----	NLCGRQQFFIDE-RLIGWNDWIIAPTGYGYNYCEGSCPAYLAFVPGSASSE		
Activin $\beta_D$ (x)	CDQNS----	NLCCKRKDYVDE-KDIGWNDWIIKPEGYQINYMGLCPMHIAGAPGMAASE		
Activin $\beta_C$ (m)	CQGAS----	RMCCCRQEEFVDF-REIGWNDWIIQPEGYAMNFCIGQCPLIHIVAGMPGISASE		
Activin $\beta_E$ (m)	CEPET----	PLCCRRKHVVDF-QELGWRDNILOPEGYQLNYCSGQCPHILAGSPGIAASE		
		CC	C	C
Activin $\beta_A$ (h)	HSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNI	IKKDIQNMI	VEECGCS	
Activin $\beta_B$ (h)	HTAVVNQYRMRGGLNPGPVN-	SCCIPTKLSSMSMLYFDDEYNI	VKRDVPNNI	VEECGCA
Activin $\beta_D$ (x)	HTTVLNLIKANNIQ-TAVN-	SCCVPTKRRPLSLMLYFDRNNV	LKTDIADMIVE	ACGCS
Activin $\beta_C$ (m)	HTAVLNLLKANAAGTTGRGSCCVETSRRPLSLLYYDRDSNIVKTDI	FDMVVE	ACGCS	
Activin $\beta_E$ (m)	HSVAFSLLKANNPEW-AG-	SSCCVPTARRPLSLLYLDHNGNVVKT	DPDMVVE	ACGCS

FIG. 3

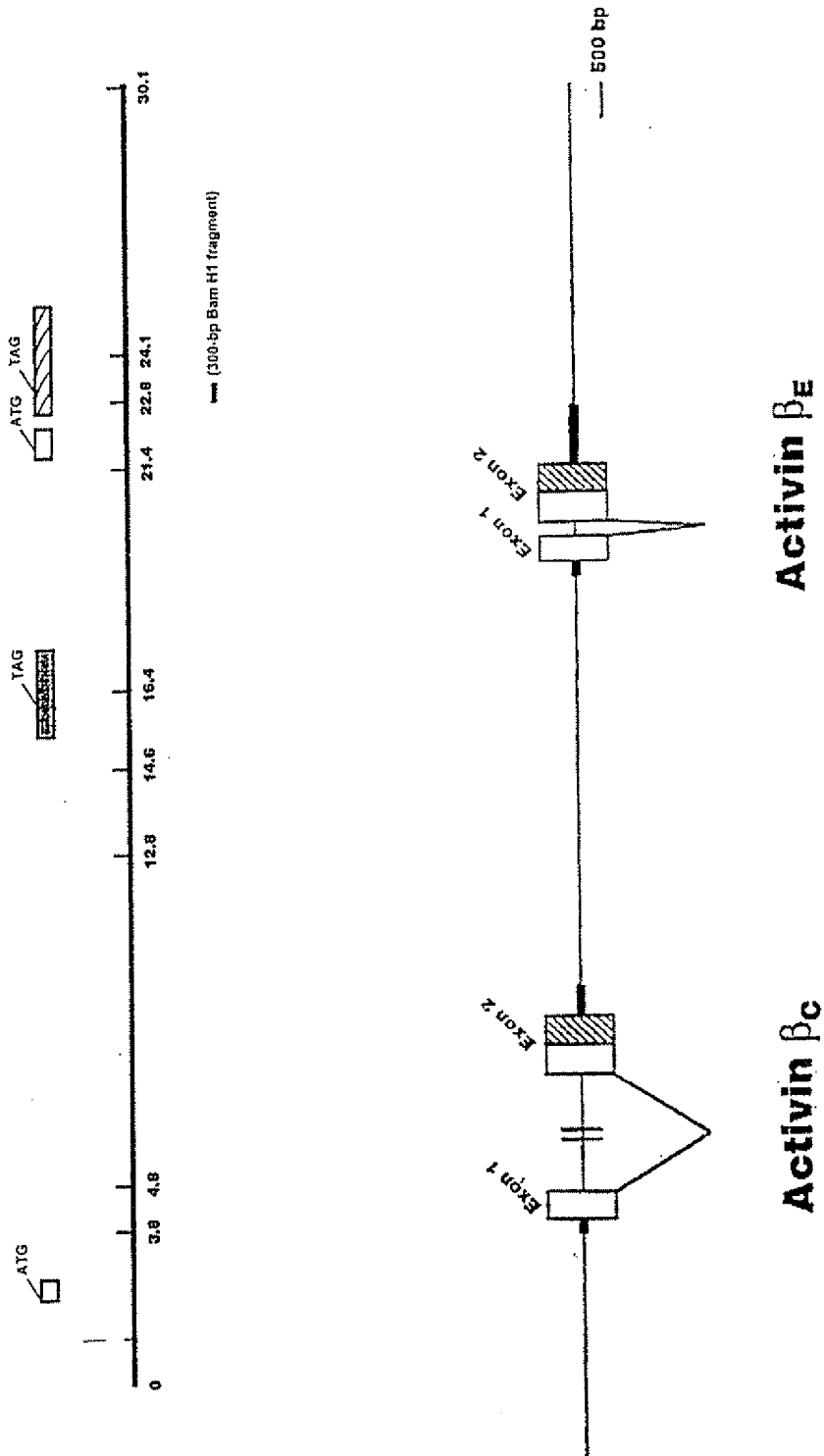


FIG. 4A

5/15

GGATCCACCTCTAAAGAGAGTCACTAGTGCACAAACAGCCTTTCTCTCTCCTGGGACATGG  
 TTGACCCAGTACACCCATCCTCAGCCTTAAGCTAGAGGCTAATCGACTGCCTACCACAAG  
 CAATGTCAATTTTGTTCCTGGCAAACACACCCTTAGCTCTCCCTTAGTCAACTATGTAATC  
 TACTCTGCCTCCCTGACCCTGCCACCGGAAGTTCTATTCCACGATGATATGCCTTAGTG  
 TCTCCCTTGAATTCTGTGGCTCTCCGAAGAACCCTTCATCAGGGTCACTGAAGATTAT 300  
 ATTGCTGCCTTCCAAAGAAAGGCTCATCTCTCAATCTGGAGACCCGAGCCAGATGTGC  
 TACTAAATAGTGTCTCTGCCCCCTTCTGCCCCCTCGGGGTGACATACCAAGTCTCATTGGT  
 TGGACATTCCTCTAGTTTCCAGCTGAATTTGACCAAAAGGAGACTCCAGTTGGAAACTAA  
 AGCCTAGGATAACACAGAAAACTCCATCAGGCATAGTGGCTCACATGGACAAGTCCAGC  
 CCTGCACACGAGAGACTGAGGCCAGCTAGCTCTAGGCTAGCCTGGGTATGCCATTAAGA 600  
 CCTTTGTACAAAGTAAAGAAGGAAGTAAGAAATATCTCCTATGTCACTTATTTAC  
 AGGCTGTGGCTCCATGGGCTGTGCAGTGTCCATGTCACATCATTTGTTTCGACGCTTAGGA  
 GTCCGATGGTGTCTCCGCTGTCTAGTCCCTGGTGTGTAACATCCCCCTTTCCAGTTCA  
 CTGTGTAGAGCAGGCTTGCTAAAACTCACAGAGATCTGCCCTTCTTCTGCTAGGATGAATG  
 GTCTTCACCGCCATGCCTAGCTTTGAGCATTATTTCTGTTGGTTCCCTTAACCCACCTG 900  
 TGACTTGGTGAATCTCTCACTAATTCCTCCTGAGATAGCTCAAAGGGTTCCCATCTCCTA  
 TCTGGATGTGCCCTAATACCCAGGTCAAGGAGCTTTCTTCATATGACCCATCTGTCCC  
 ATACCTCACGCCCCCTTCTCCCGCAGTTGCCCTTCTTCCCTCCTCTCCCTCCATCCTCC  
 ATTCAGAACTACCTTCCAACAGTTTCCAGGCCTAATAATGAAGTACTGCTTTTGTGGATCT  
 CATCTCCCTCTGTACTCACTCCAGAGATGAGAGATCAATAATCAATTAACAGTGAGC 1200  
 CAGCACTGGCCACAGGTTGGGGGGCAGAGCATTCTCAGAGCTGGTGGCTGGAAGTCTGC  
 CCACCTCCCTTCCACATGCTCTTAGAGCATTACCATGAATCTGCCAGAGGCTTGAGTG  
 GGACGAGAAAAGGAAGGCCCTCCAATAATGAGAGGGGCATTATCCGAGATCTGGTGCTC  
 TTTGACCTTTGCTTTCTTTCTTCCCGCCCCCTTTGGCCGAACAGCCCTGAACCATGGGG  
 TCGATATTGTCTGCAGCCCAAGGCCAGGTTTGGCGAAAGCCCATGTCTCCTCTGGGAAAT 1500  
 GTGATGTCTGTGTTTGTCTCAGCACATGCTCCTTTTCTTACCAGGTAAGAGGTCTCCGTAA  
 TGGAGATGCAGAGAGGCCACCTGCAAGCGATCTTTTGGGGAGCTGCCAGTCAGAGCTGT  
 GGGGTGTTTCCCAACCCTTGACTTGTCTGCCCTCCATCTGAAGAGGTTCCATGCTTTCTCT  
 TCCTCCCCCTTCTGCCAGTGTCTCCACTTTTATCCCTCTCTCAGATCAAACCTTCTTCCC  
 TTAGACTGACAGTTTTCGGTGCCAGGACCAAGACCAGGGCAGGGAAAAGTGAGGCATTCA 1800  
 TCCGACAGCAGAAATTTTAAGGTGGGGGCAACAACATCATCAGTGAGGAAAACCGGTATTT  
 TAATGAAAGATTTTTCAGAGTGCATGCTGCAAGGAACAAATAGCATATTTAAAAATAA  
 GCACCCGTCGGTGTATTCATTTTCTTTCTTTTGGCTCTGCTTTTGTATTTAAAAAA  
 AAAGAAGAAGAAGAAGAAGAAAATGGAATGCTCTGTCTCTTCTTCCACCTTCTCCCT  
 GAACCCCTGACCCCAAGACTCCTTCAGGCTGTCTCCTTTGGGCAATAAGTAGTCTCTGC 2100  
 TTCTTCTATTTCTTCCCTTCCCGTTCTCAAACCCGATGCTACCCCCCAACCTCCAGTCAGCCT  
 GGTGGCAAGGCGGGCAGTGTGGCAGATGCCCTGGGTATTTATAAACTGGAAGCACTTCTTG  
 GGGAAAGTGACTAAGATGCTAAGATCGTATTTATAGCTGAGTTCTGACGTAAGTGTACG  
 GGGAGGTAGGACCGGGCCAGGCGCAACAGCAAGAGGGTGGAAGAGCTAGAAGCCATG  
 GGCACCAGACTGGGCTGGGGAAGGTAGCGTCCGTGTCTCTGTCAGCCACCTCTTTTCGACT 2400  
 TCCCCTGAGCCTGTTCCTGTCACAAGCCCTCCTAGACACCCAGGAAGGGGCAGTTGCAG  
 TCTCATCGGTCAITTCCTCAAGGGGCCAACAAGCTGTCACTCTCCTGTCAGCAACCAACAGA  
 GAGAGACCTGAGAGCTCTAGGGAGCAGTAGCCAAAACCTTGAATATGTTTCAGGGCCTG  
 AGGAGAAAAGCCAGTGGATACAAATGTCTCCTAATCCTTTCTCCCTCTTCTCCGCTCCAT  
 GGTGCCCTGAGTTTCTAGGAAGTCTGGCCTCCGACATCAGGCTCCATGGCCTCGTGTCAA 2700  
 TGGTGGTAGGGATGCACTCTGGCAGTAGCGAGGTAAAAGAGCAGAGGAGCTAGCCCTTGT  
 CTACTGCCCTGTGAGGGAGGCAAGATCCTAAGGGAGTCCATGAGGCATATCCACCATCTTT  
 CCACTCTCCAGCAGCTCTGTCCCTGTCCCCAGGATTCTCTGGTCAACCACTGTCCAGTGA  
 CAAGCACTGCCCCATGGTGGACACAGAAGGTACTGAGCTCAGCCTTCAAACACCCCTTT  
 CCCCTGGTCCAGATCTAGAATCTCTAGGCAGCACCCGCGAGCTAAAGAAGGGAAAGGCAG 3000  
 AATGGTCCGGGGGCTTAAGTACACCCCTCTTTGTCCACAGCTCTCTCCCTAATGCCACC  
 CATGTTCTTAAACAAACCTGGCACCTGGAGAAAGGCTAGCCTGATAGTATCTCTTGTATC  
 AGAGGTAGAGGGTCACTCTCATTTAAGATCATGCATCTCAGGGCTGGCTACGTAGCCAG  
 CGGTAGGGTAATAGCCTAGCATGCACAAAGCCCTGGGTTCATCTCTGAGCAGCAATAAA

FIG. 4B

AGCCAGGTATGCTGAGGGTGGTACATGCCTTTAATCCCAGCACTCAAGAATAGACGTTGA 3300  
AGGTTACCCCTTAACAACACAGGGAATTGAAGGTCACCCCTTGACTACACAGGGAATTCAAA  
GTCAGCCTGGGCTACATACAGGAGGTCTGTCTCAGACCACAACAAAATAAAGTAAITCA  
CCATAGTCTTCACGACAAAGGAACTGAGGCATGAGAATCACTTATACTAGAAGTTCTAG  
CCAGCCTGGACATCGTAGAAACACCTCATCTCAAAAGACAAAAACCAAAGCAAATTAATA  
AATTAATAAATAACAACCCACACCCCCACACCCCAAAATAAGCTGGGTGCTGATGGCATA 3600  
ATCTTTAATGCCAACATTTCAGGAGGCAGAGGCAGGTGGAGCTCTGTGAGTCCAAGGCCAG  
CCTGGTCTACAGAGCAAGTTCAGGACAGCCAGGGCTACACAAAGAGAGCCTATAATGGG  
GTGGGGGAGGTGAGATGTTGGGAGATAACTCAGTAGATAAAGCTATTGCTTTACAAGCG  
CATGGACCTGCATTAGAGTCTCCAGAGCCCAGGTAGAAATGCAATCCCAGCGTTGCAGAC  
ACTGGGACTCACTGGCCAGCCAGTGTAGTCTAATTAGTGAGTTCCAGACCAGCAAGGATG 3900  
TCCTGTCTCAAAGGAGGTAGGTGGATGACGTCCTAGGGATGGCACACAGGGCTGTGCTC  
CAGCTTACACATGCACACACACAGCACACACACGTCACACAAGCACACACATGCACAC  
ACACGAGCACGCGGTACACACACACACATGCGCACACATTAAAAACAAAGTCGGGG  
GGCTGGAGAGATGGCTCAGTGGCTAAGAGGCACTGGCTGCTCTTTTCAGGGGACTTGAGTT  
CAATCCCAGCAACACGTTGTGGCTCACAACCATCTGTAATGGCATCCAATGCCCTGCTT 4200  
CTGGTGTATCTGATTGAAGACAGTGACAGTGTACTCACATATATAAAATAAATAAATCTT  
TCAAACAAACAAGCAAAAATAAAACAAAGCCTGGATGACCAAGACCTGAGGCGGCTTTTC  
CGGCTTTGTCCCCTAGGTCCCTTAAATTTCCATTTTCCAACCTACATTACCAGAAGCCTGG  
CACCCTCCTGGGCTGCCTCAGATTGGCCAACTCCTACAGTACCCTAAAGGCAGTAGTT  
ACCAGGCACAGGTGCTTCAAAGCGCTCGCCCTTCTGCCAGCTCTGCCGGACTCTGCTCTG 4500  
CTGTAAGATTGCATCACCACGAGCCACCTGGCCGGGTTTCCCCAGACATCACAGAAGAT  
GTCTCATCCCTGCTCTGGCCCTCCCCCAGGTGGACTCTGGGCAGCCAGCACAGGCAGGG  
TCAAAGGTAATTTCTATGTCCTCGCACTGCCACCTAGGCCTGATCAAGAGGACTCCATT  
TTTTTTTTTTAAACCACCTTTCTAAACCTAATCGGCTTGAACGCTAGAATTATTGTT  
CTGACATGGAAACCCCGAGAACTAACCCACTGAAACAGCCTCATAAAACGGTTTCGTTTT 4800  
CAAGCTCGGAAGTCAGTCAGGGCATACTGAGGAGCCACCGCCCATCCCCACCTGGCTCCT  
ATGGACTCCCTCACCTGAATCCAGACCTTGGGAAGTACCGGTGTGAATCATCCTCTTT  
CTTGCAATCATGCATGTCCACGTTGCACCTCCATACCCCTACCTCAAGCCACCACACCCAG  
ATGGTGAAACTCGAGTCCCCGGGGAGCCCTCCACCTCGAGGGGCTGATGGGCAGCGCAG  
CTGGCCAATCCTAAGGTTCAAGAGGTAGGTCTGCTGGTTGACCGCTAGGTGAGGGATCC 5099

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GGACACACTAAGAAGCATCTGGTTTGACATTCTTTCTCTCCCTTTTCCTTAACCTTTCAG		
ATGGTAACCTGTGACTTAGTGGGCAGAAATCCAACCTGAACTAACAAAGAAAGGTTGGGTGAA		
AGCCCTAAGGAGGAAATCCGAGCAGTTGGGAAGCAGAGGTGGCCTGGTCTATAGAGTTA		
ATTCCAGGGATACATAGGAAACCTGTCTCAAAAAAAGAAAAGCAAAGCAAAGCAAAG		
CAAAGCAAAGCAAAGAAAAGAAAAGAGAAGTGGAGGAGGAGGGGAGGAGGAGTGGGG		
AGGAGAAGCGAGGAAGAGGAAACTTTAAAGTGCCTCTTTTTTTTTTTTTTTTTTTTAA		
GAGTCAACCATTTAAGCAGGTGTGGTGACATATACITTTTATCTCAGCACTTACAAGACAG		
ACACAGGTAGATCTCTGTGAGTTCAAGACTAGGAGGGTCTACATAGCCAGITCCAGGCCA		
GTCAGGGCTACACCATGAGATCCAGTCTCAAAAACAACCAAGCAAGCAACAACAAAGAAG		
TCAGCCTCTTAACCTGGACACGGTAGTCCATGCCTGTGAGACCAGATTGAGAAGCAGATG		
CAGAAGTTCAAAACCAACCTCCACTATATAGCAAGATCAAGTAACCCAAGCTGAGTGC		
AAATCTGACTCAACCACACACCATGCACACACAAAAGTCACAATTTTTTTTTTCTTTT		
GAGACTGTTAAGTCTGACTTCTGGTGATTATCCAGCCTTAGCCCCGTAAGTACCGCTGC		
TCACAACACTTTTCCCCCAGGCTTCACAGCAAGGCTCTAACCTTTGTCCTCTGCGTCTGG		
CAGCTCATTTCCGCTAACAATCTCCCAACAGAAGCAAGAGGGGAGACATTTCTTTCTCA		
AACAATCCACACAACCCCTCTGTACTTTCACTGGGCTACAGGAGAATACTGCCAACGTC		
CACCTGAAGTGTCTCATTTTCACGCCAGATGTTGGCCGCTGTATCTCTGACAAAAGGA		
GTCAATGCCAGTCGGAGSTCAGTCAATTCCTCCAGGGTCCCTGGTGGCCAGGACAGAGT		
TGAAGCCACTCCCGTTGAGACCCCTGAATATAGGCTTTGGGTCTTTAAGGAGGCTATCCT		
CCAGCAATGGCCCTCCTCTCTGCTCTCTGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT		
CCAGCAATGGCCCTCCTCTCTGCTCTCTGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT		
M A S S L L L A L L F L T P T T V V	16	Activi
AACCCCAAAGCTGAGGGTCCATGCCAGCATGTTGGGGTGCCATCTTTGACCTGGAGAGC		
N P K T E G P C P A C W G A I F D L E S	38	Exon
CAGCGGGAGCTGCTTCTCGATTGCGCCAGGAAAGTATCCTGGACAGCTGCACCTCAGC		
Q R E L L D L A K K S I L D K L H L S	56	
CAGCGCCCACTACTCAGTCGGCCAGTGTCCAGAGGGGCTCTCAAGACCGCGCTGCAGCGC		
Q R P I L S R P V S R G A L K T A L Q R	78	
CTCCGCGGCTCGACGGGAAACCTGTTGGAGCATGACCAGAGACAAGAAGAATATGAG		
L R G P R R E T L L E H D Q R Q E E Y E	98	
ATCATCAGCTTTGCTGACACAGGTAGGTCCATGGTCCCTAGCTCTGGCCCCAAACCTGAC		
I I S F A D T	105	
CCTAGGAAGGAGAAAGTCTCTCTCTCTAGTTAGCTTCTGACTTCATCCTCGCCAAAACAT		
CCTGCTTGACACTCTGCGCCTGCGCACCCGGGGGGCTCTACGCTCTAGGTTCTCTAAG		
TTAGGTCAAGTTCACTTGGTGGAGCTGGGAGCTGTGGTTTCACTTCTTTAGAAATCTC		
TCT		
Activin beta C intron (12-kb)-----GTATTTTAAACCTATAAC		
CCCAGCATATGAAGTAGGGGAGGGGACCCCATCAGTTCAAGGTTGTCTTGACTGCA		
TATTGAGTGTAAAGCTAGCTTGGGCAGCATGAGCCATCTCTCAATCAAAGGGAGAGAA		
AGGTATGCTGAGAACAGAGGCGCTGGTCCAGCAGACACTCCAAGGCTGCGATAACTTC		
CCTGTGCTTCTTGATTTTAAACAGACCTCTCCAGCATCAACCAGACCCGCTCGAGTTC		
D L S S I N Q T R L E F	117	Activi
CACCTTCTCTGCTAGAATGGCCAGTGGCATGGAGGTCCGGCAGACCCGCTTCATGTTCTTC		
H F S G R M A S G M E V R Q T R F M F F	137	Exon :
GTGCACTTCCCCACAATGCCACCCAGACCATGAATATAAGAGTTCTTGTGCTAAGACCA		
V Q F P H N A T Q T M N I R V L V L R P	157	
TATGACACCAACCTCACCTTGACAAGTCACTACGTGCTGCAAGTGAATGCCAGTGGCTGG		
Y D T N L T L T S Q Y V V Q V N A S G W	177	
TACCAGCTTCTCTGGGACCTGAAGCTCAAGCTGCTTGCCAGCCAGGGACACCTTACTCTG		
Y Q L L L G P E A Q A A C S Q G H L T L	197	
GAGCTGTTACAGAAAGCCAGGTGGCCACAGTTCCTTGATCCTGGGCTGGTTTCCCAC		
E L V P E S Q V A H S S L I L G W F S H	217	
AGGCCCTTTGTGGCAGCCAGGTAAAGGGTTGAGGCCAAGCATGGGTTTGGCCGCGAGGT		
R P F V A A Q V R V E G K H R V R R R G	237	
ATCGATTGCCAGGGGGGTCCAGGATGTGCTGTCGACAAGAGTTTTTTGTAGACTTCCGT		

FIG. 4 D

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I D C Q G G S R M C C R Q E F F V D F R 257  
 GAGATTGGCTGGAATGACTGGATCATCCAGCCTGAAGGCTATGCCATGAACCTCTGCACT  
 E I G W N D W I I Q P E G Y A M N F C T 277  
 GGGCAGTGGCCACTACATGTGGCAGGCATGCCTGGCATCTCTGCCCTCCTTTCACACTGCA  
 G Q C P L H V A G M P G I S A S F H T A 297  
 GTGCTGAATCTGCTCAAAGCCACGCAGCTGCTGGCACCCTGGCAGGGGCTCGTGCTGC  
 V L N L L K A N A A A G T T G R G S C C 317  
 GTGCCTACATCTCGCCCCCTCTCTCTTTGCTCTACTATGACAGGGACAGCAACATTTGTC  
 V P T S R R P L S L L Y Y D R D S N I V 337  
 AAGACGGATATACCTGACATGGTGGTGGAGGCTGGGGGTGTAGTTAGCTTATGGGTGAT  
 K T D I P D M V V E A C G C S \* 352  
 ACAGGCTGCTGAGGTAGAATGGCCTTCCCTCAGGAAGGGGAAACTCTGTTCCTCCTCTG  
 TCCAGAATGGAAACACCTTTCTAAGCATGCAGACATCCCTCTGTGGACTTCAGGGGATCC  
 ACCTCTAAGAGAGTCACTAGTGCACAACAGCCTTTCTCTCTCTGGGACATGGTTGACC  
 CAGTACCCCATCCTCAGCCTTAAGCTAGAGGCTAATCGACTGCCCTACCACAAGCAATGT  
 CATTTTGTTCCTGGCAAACACACCCTTAGCTCTCCCTTAGTCAACTATGTAACTACTCT  
 CCTCCTGACCCCTGCCACCGGAAGTTCCCTATTCACGATGATATGCCCTTAGTGTCTCCC  
 CTTGAATTTCTGTGGCTCTCCGAAGAACCCCTTCATCAGGGTCACTGAAGATTATATTGCT  
 GCCTTCCAAAGAAAGGCTCATCTCTCAAATCTGGAGACCCGAGCCCATGTGCTACTAA  
 ATAGTCTTCTCTGCCCTCTCTGCCCTCGGGGTGACATACCAAGTCTCATTTGGTTGGACA  
 TTCTCTAGTTTCCAGCTGAATTTGACCAAAGGAGACTCCAGTTGGAACATAAAGCCTA  
 GGATAACACAGAAAACCTCCATCAGGCATAGTGGCTCAGATGGACAAGTCCAGCCCTGCA  
 CAGGAGAGACTGAGGCCAGCTAGCTCTAGGCTAGCCTGGGTTATGCCATTAGACCTTTG  
 TCACAAAGTAAAGAAGGAAGAATGAAGAATATCTCTATGTCACTTATTACAGGCTG  
 TGGCTCCATGGGCTGTGCACTGTCCATGTCTCATCATTTGTTTCGACGCTTAGGAGTCCGA  
 TGGTGTCTCCGCTGTCTAGTCCCTGGTGTGTTGAACATCCCCCTTTCCAGTTCACTGTGT  
 AGAGCAGGCTTGTCTAAACTCACAGAGATCTGCCTTCTCTCTAGGATGAATGGTCTTC  
 ACCGCCATGCCCTAGCTTTGAGCATTATTTCTGTGGTTCCCTTAAACCCACCTGTGACTT  
 GGTGACTCTCTCACTAATTCCTCCTGAGATAGCTCAAAGGGTTCCCATCTCTCTATCTGA  
 TGTGCCCTAATACCCAGGTCACAGGAGCTTTCTTCATATGACCCATCTGTCCCATACCT  
 CACGCCCTTCTCTCCCGCAGTTGCCCTTCTTCCCTCTCTCCCTCCATCCCTCCATTGAG  
 AACTACCTTCCAACAGTTTCCAGGCTAATAATGAAGTACCTTTTGGTGGATCTCATCTC  
 CCTCTGTACTCACTCCAAGAGATGAGAGAGATCAATAATCAATTACAGTGAGCCAGCAC  
 TGGCCACAGGTTGGGGGGCAGAGGCTTTCTCAGAGCTGGTGGCTGGAACTCTGCCACCT  
 CCTTCCACATGCTCTTAGAGCATTACCATGAATCCTGCCAGAGGCTTGAAGTGGGACGA  
 GAAAAGGAAGGCCCTCCAATAATGAGAGGCGGCATTATCCAGATCTGGTGTCTCTTGAC  
 CTTTGCTCTTCTTCTTCTTCCGCCCTTTGGCCGAACAGCCCTGAACCATGGGTCGATA  
 TTGTCTGCAGCCCAAGGCCAGGTTTGGCCAAAGCCCATGTCTCTCTGGGAAATGTGATG  
 TCTGTGTTTGTCTCAGCAGTCTCTCTTTTCCCTACCAGGTAAGAGGCTCTCCGTAATGGAGA  
 TGCAGAGAGGCCACCTGCAAGCGATCTTTTGGGGAGCTGCGAGTCAAGAGCTGTGGGGTG  
 TTTCCACCCCTTGACTTGTCTGCCCTCCATCTGAAGAGGTTCCATGTTTCTCTCTCTCC  
 CCTTCTGCCAGTGTGTCCCACTTTTATCCCTCTCTCAGATCAAACCTTCTTCCCTTAGAC  
 TGACAGTTTCGGTGGCCAGGACCAAGACCAGGGCAGGGGAAAAGTGAGGCATTCTATCCGAC  
 AGCAGAAATTTAAGGTGGGGGCAACCAACTCATCAGTGGAGGAAACCGGTATTTTAAATGA  
 AAGATTTTTCAGAGTGGATGCTGCAAGGAACAAAATAGCATATTTAAAAATAAGCACCC  
 GTCCGTGTTATTCATTTCTTTCTTTTGGCTCTGCTTTTGTATTTAAAAAAAAGAA  
 GAAGAAGAAAGAAAAATGGAATGCTCTGTCTCTTCTTCCGACCTCTCTCCCTGAACCC  
 CTGACCCCAAGACTCTTTCAGGCTGTCTCTCTTTTGGCCAAATAAGTAGTCTCTCTCTTC  
 ZATTTCTTTCCCGTTCTCAAACCCGATGCTACCCCCCAACCTCCAGTCAAGCCTGGTGGC  
 AAGCGGGGCAAGTGTGGCAGATGCCCTGGGTATTTATAAAGTGAAGCACTCTTTGGGGAAA  
 GTGACTAAGATGCTAAGATCGTATTTATAGCTGAGTTCTGACGTAAGTGTCAAGCGGGAG  
 GTAGGACCGGGCCAGGCGCAACAGCAAGAGGGGTGGGAAGAGCTAGAAGCCATGGGCACC  
 AGACTGGGCTGGGGAAGGTAGCGTCTCTCTCTCTGAGCCACCTCTTTGACTTCCCT  
 GAGCCTGTTTCTGCAACAGCCCTCTAGACACCCAGGAAGGGGCACTTGCAGTCTCAT

$\beta_E$  Promoter  
 Region

FIG. 4E

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CGGTGATTCTCCAAGGGGCAACAAGCTGTCTACTCTCTCTGACGACCAACACAGAGAGAGA  
CCTGAGAGCTCTTAGGGAGCAGTAGCCAAAACCTTTGAATATGTTTCAGGGCCCTGAGGAGA  
AAGAGCAGTGGATACAAATGTCTCTTAATCTTTCTCCCTCTTCTCCGCTCCATGGTGGC  
CTGAGTTTTCTAGGAAGTCTGGCCTCCGACATCAGGCTCCATGGCCCTGCTGTCGAATGGTGG  
TAGGGATGCACCTCTGGCAGTAGCGAGGTAAAAGAGCAGAGGAGCTAGCCCTTGTCTACTG  
CCTGTGAGGGAGGCAAGATCCTAAGGGAGTCCATGAGGCATATCCACCATCTTTCCACTC  
TCCAGCAGCTCTGTCCCTGTCCCCAGGATCTCTGCTGGTCAACCACCTAGTCCCAAGCAAGCA  
CTGCCCCATGCTGCACACAGAAAGCTATGAGCTAGCCTTCAAAACACCCCTCTTCCCCCTG  
GTCCAGACTCTAGAATCTCTAGGCAGCACCGCCAGCTAAAGAAAGGAAAGGCAGAATGGT  
CGGGGGCCCTTTAAGTCACTCCCTCTTTTGTCCACAGCTCTCTCCCTAATGCCACCCATGTT  
CTTAAACAAACCTGGCACCTGGAGAAAGGCTAGCCTGATAGTATCCTCTGTATCAGAGGT  
AGAGGGTCACTCTCATTTAAGATCATGCATCTCAGGGCTGGCTACGTAGCCAGCGGTAG  
GGTAATAGCCTAGCATGCACAAAGCCCTGGGTTCAATCCTGAGCACCGAATAAAAGCCAG  
GTATGCTGAGGGTGTATCATGCCTTTAATCCAGCACTCAAGAATAGACGTTGAAGGTTA  
CCCTTAAACACACAGGGAATTGAAGGTCAACCTTGACTACACAGGGAATTCAAAGCTCAGC  
CTGGGCTACATACAGGAGAGTCTGTCTCAGACCACAAATAAAGATTAATTCACCATAG  
TCTTCACGACAAAGAAACTGAGGCATGAGATCACTTATACTAGAAGTTCTAGCCAGCC  
TGGACATCGTAGAAACACCTCATCTCAAAAGACAAAAACCAAGCAATTAATAAAATTA  
AATAATACAAACCCACACCCCCACACCCCAATAAGCTGGGTCTGATGGCATACTCTTT  
AATGCCAACATTACAGGAGCCAGAGGCCAGGTGGAGCTCTGTGAGTCCAGGCCAGCCCTGGT  
CTACAGAGCAAGTTCACAGGACAGCCAGGGGCTACACAAAGAGAGCCATATATGGGGTGGG  
GGAGGTGAGATGTTGGGAGATAACTCAGTAGATAAAGCTATTGCTTTACAAGCCGATGGA  
CCTGCAATTAGAGTCTCCAGAGCCCAAGGTAGAAATGCAATCCAGGCTTGCAGAGCTGGG  
ACTCACTGSCCAGCCAGTGTAGTCTAATTAGTAGGTTCCAGACCGCAAGCATGTCCTGT  
CTCAAAGGAGGATAGGTGGATGACGTCCTTAGGGATGGCACACAGGGCTGTGCTCCAGCTT  
ACACATGACACACACAGGACACACACACAGTGCACACACAGCACACACATGCACACACAGCA  
GCACGCGCGTACACACACACACACATGCGCACACATTAAAAACAAAGTCCGGGGGGCTGG  
AGAGATGGCTCAGTGGCTAAGAGGCACTGGTGTCTTTTCAGGGGACTTGAGTTCAATTC  
CCAGCAACACGTTGTGGCTCACAAACCATCTGTATAGGATCCAATGCCCTGCTTCTGGTG  
TATCTGATTGAAGACAGTGACAGTGTACTCAGATATATAAAATATAAATCTTTCAAC  
AAACAAAGCAAAATAAAACAAAGCCCTGGATGACCAAGACCTGAGGCGGCTTTTCCGGCTT  
TGTCCCTTAGGTCCCTTAAATTTCCATTTTCCAATACATAGCACAGAAGCTTGGCACCAC  
TCTTGGGCTGCTCAGATTTGGCCAACTCTCTCAGTACCTTAAAGGCAGTAGTTACCAGG  
CACAGGTGCTTTCAAAGCGCTGCCCCCTGTGCGAGCTCTGCGGACTCTGCTCTGCTGTAA  
GATTGCATACCAACGAGGCCACCTGGCCGGGTTTCCCCAGACATCAGAGAAGATGTCCTC  
ATCCCTGCTCTGCCCCCTCCCCAGGTGGACTCTGGGCAGCCAGCACAGGCAGGGTCAAAA  
GGTAATTTCTATGTCTCGCACTGCCACCTAGGCTGATCAAGAGGACTCCATTTTTTTTT  
TTTTAAACCAACCTTTCTAAACCTAATCTGGCTTGAACGCTAGAATTATTGTTCTGACA  
TGGAAACCCCGAGACTAACCCTAGTAAACAGCCCTATAAAACGGTTTCTGTTTCAAGCT  
CGGAAGTCAGTCAGGGCATACTGAGGAGCCACCGCCCCATCCCCACTGGCTCTTATAGGAC  
TCCCTCACCTGAATCCAGACCTTGGGAATCAGCGTGAATATCATCTCTTTCTTGGCA  
TCATGTCATGTCACGTTGGCACTCTCATCCCTTACCTCAAGCCACCACACCCAGATGGTG  
AACTCGAGTCCCGGGGAGCCCTCCACCTCGAGGGGCTGATGGGCAGCCAGCTGGCC  
AATCTTAAGGTTACAGAGGTTAGGTCTGCTGGTTGACCGCTAGGTGAGGGATCCAAGCAAC  
TGGCTCTAAAGAGGCCCTGCAGTAGTTAGTCATGAAGTGTAGGGTCCACATAGCTTAC  
CCACAGGCGATCTACTCTCAGTCTTCTGAGTCTTAGGCTATTGAGGACAAGTAGCTTG  
GTCTGCTCTTTGTCAAGGGTAGCTGTGACACTGTTTGTCTGTTGCTGCTGCTGCTGCTG  
TGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG  
CACCCATTGAGGTTCTCAAAGCAGAGCCATCTACCTGGAGCATGAGCTTCCAAAGCC

M K L P K A  
CAGCTCTGGCTAATACTGCTCTGGGCATTGCTGTGGGTGCAGAGTAGAAGATCTGCGTGC  
Q L W L I L L W A L V W V Q S R R S A C  
CCGTCTCTGTGGGGGCCAACACTGGCACCCCAAGGAGAACCGCTCTGCTCTGGAGCTA  
P S C G G P T L A P Q G E R A L V L E L

6  
26  
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Activin  $\beta_E$   
Exon 1

FIG. 4F

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GCGCAAGACGCAAAATCTTGGAGGGAGCTGCACCTTAACCAGCCCTCCCCAGAATAACTCGGCTC  
 A K Q Q I L E G L H L T S R P R I T R 66  
 CTGCCCCAGGCAGCACTGACCAGAGCCCTCCCGAGACTGCAGCCCCAGAGCATGGTCCCTC  
 L P Q A A L T R A L R R L Q P K S M V P 86  
 GGCAACCGAGAGAAAGTCATCAGCTTTGCTACCATCATAGGTGGGTGAGGGGAGAGAGAC  
 G N R E K V I S F A T I I 99  
 CGGGGACAGGAGACACAAGGAGAGGCTGCAGGGGAGGACAGGAGGCGTGGGGTGTGGCAAG  
 AGAAAGAGCTGGGGACACAGCCGTCAGGCCCTTTGCAGAAGGTCGTATGAGAGAATG  
 Activin beta E intron (236-bp)  
 AAGGGATGTTGGGTAGGGTGGCCGAGTGCAGAGACAAGCTTTGTTTCATGTTTAAATTT  
 GCTTTTGTGTTTGTGTTTGTGTTTGTGTTTGTGCTGACAGACAAATCCACTTCAACCTACCGC  
 D K S T S T Y R 107  
 TCCATGCTCACCCTCCAGCTGTCCCCCTCTTTGGTCCCAACCCTGTACCATGCCCCGCTC  
 S M L T F Q L S P L W S H L Y H A R L 127  
 TGGTTGCATGTGCCTCCCTCTTTTCCGGGCACTCTGTACCTGAGGATCTTCCGTTGCGGC  
 W L H V P P S F P G T L Y L R I F R C G 147  
 ACCACTAGGTGCGGAGGATTCCGCACCTTCTAGCTGAGCACCACCACTTCTCTGCGC  
 T T R C R G F R T F L A E H Q T T S S G 167  
 TGGCAGCCCTGACTCTGCCCTCTAGCGGCTTGGGAGTGAGGACTCTGGCGTCTGAAA  
 W H A L T L P S S G L R S E D S G V V K 187  
 CTCCAACCTGGAATTTAGACCCCTTGACCTTAACRSCACCCGCTGCGGAGCTGCCAGCGCTG  
 L Q L E F R P L D L N S T A A G L P R L 207  
 CTCTTGGACACAGCGGGACAGCAACGTCCTTCTTGAACCTTAAGATCCGAGCTAATGAA  
 L L D T A G Q Q R P F L E L K I R A N E 227  
 CCTGGAGCAGGTGCGGCCAGGAGGAGGACTCCACCTGTGAGCCTGAGACCCCTTATGT  
 P G A G R A R R R T P T C E P E T P L C 247  
 TGTAGCGAGACCACTATGTAGACTTCCAGGAGCTGGGGTGGCGGATGGATCCTGCAG  
 C R R D H Y V D F Q E L G W R D W I L Q 267  
 CCGGAGGGATACAGCTGAATTACTGCASTGGGCGAGTCCCGCCACCTTGGCTGGCAGT  
 P E G Y Q L N Y C S G Q C P P H L A G S 287  
 CCTGATCTGCTGCTTCCATTCTGCCGCTCTTLAGCTCCTCAAAGCCACACACCT  
 P G I A A S F H S A V F S L L K A N N P 307  
 TGGCCTGCGGGTCTTCTGCTGTGTCCTCCACTGCACGAAGGCTCTCTCTCTCTCTAC  
 W P A G S S C C V P T A R R F L S L L Y 327  
 CTTGACCATAATGGCAATGTGGTCAAGACCGATGTGCCAGACATGGTAGTAGAGGCTGT  
 L D H N G N V V K T D V P D M V V E A C 347  
 GGCTGCAGCTAGCAACAGGGCTGAAGGTTCTGGGTGAAGTTCAAGGTTCAAGTTGGGGG  
 G C S \* 350  
 TTCCACGCTGTCTGGAAGCTCGAGTTCGGATCCATACTGACACCCCAACAGCTGTGTAG  
 CAGTATGCCTGGGTTTGACCCCTATGGAACCTAAATGGGCGTTTTCTGTCCAGATTCT  
 GGCCTATTTTCAGGCTGTTTCAAATGTGGACAGATGGGTAAAGCCGTTGCCCTTCAAGGGA  
 CTGCCCTGGCCAGCACCATTCTTACATCAAGCCCTGTTCCAGGACAGCAGGGATGCCGTG  
 GGAGGGAAGGAAGACACAGGGAGAACTATTAGTCTCTCCCGAGAAAGAAGTTCCCTCA  
 AGTAATGAAGCGGGAAGTAGAAGGTTGGGCAGATTAGGAAAAGACAAACATACAGGCTAA  
 GAACAGGGTGCATTGCTGCTTTGACAAGGTCAAGAGGAAGGAGCAGCGCCGAGGA  
 AGGAGGGGTGTCGGGGGTGCTTGGAACTCGAGAACTCAGTAAAGAGGCTGCTGAACCTCGTA  
 AGTTCTTAGGCTTCCCTCCGAGGACAGGACCAACCGGGTGACATACATTTTATATTTT  
 CTTAATAAAAAGGAGAAAGAAAAGCACCCAGAGAATTGTGTAAGGGGCCGCCAAATGGGC  
 CAGAAGCGAAGTGTGGTTTGGGAACCTCTGTGCCCCAGCGGCTTCTGAGACTTTCTCAGG  
 GGTTTTCAAGACTATTTTCATAATCACACTGAGATGTTATTTATCATTTGCTACCATTTGT  
 CTTTACATGTGACAGTGGGAACAGGGTGTGGTGGCTTACACTTATAACTACAGCACCGTG  
 AGTTCAAGACCGGCCCTTCATAGTGAATTTCTGGGCTAGCCCTGAGCTATAGAATGAGATCCT  
 GTCTCAAAGCAAACAACAACAACAACAACAACCAGACAGTCTTCCAGAGTGTGTAT  
 ATGACACAGCTGATGACATGCTGATATAGTCTTTTCTCTTCTCTCTTAAACAAT  
 TTTATTTATTTATTTATGCTGATAGTCTTTTGTCTGATGTTAAGTGCAGCGCTGG  
 CTTGCAATGGCCGTGGAGTTCAGAAGAGGGCCGCTAGATCGAGTCCGACTCCCTTAGTGA  
 GGGTAACTGAGCCTGGCGCTCTAGAACTAGTGGATCC

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## Activin Intro

## Activin Exon 2

Downst  
Activir

FIG. 4 G

11/15

G AAT TCC GGC AAT CAG ACT CAA CAG ACG GAG CAA CTG CCA TCC GAG GCT CCT GAA CCA  
GGG

CCA TTC ACC AGG AGC ATG CGG CTC CCT GAT GTC CAG CTC TGG CTG GTG CTG TGG GCA 1  
H R L P D V Q L W L V L L W A

CTG GTG CGA GCA CAG GGG ACA GGG TCT GTG TGT CCC TCC TGT GGG GGC TCC AAA CTG GCA 31  
L V R A Q G T G S V C P S C G G S K L A

CCC CAA GCA GAA CGA GCT CTG GTG CTG GAG CTA GCC AAG CAG CAA ATC CTG GAT GGG TTG 51  
F Q A E R A L V L E L A K Q Q L L D G L

CAC CTG ACC AGT CGT CCC AGA ATA ACT CAT CCT CCA CCC CAG GCA GCC GTG ACC AGA GCC 75  
H L T S R P R I T H P P P Q A A V T R A

CTC CGG AGA CTA CAG CCA GGG AGT GTG GCT CCA GGG AAT GGG GAG GAG GTC ATC AGC TTT 95  
L R R L Q F G S V A P G N G E E V I S F

GCT ACT GTC ACA GAC TCC ACT TCA GCC TAC AGC TCC CTG CTC ACT TTT CAC CTG TCC ACT 115  
A T V T D S T S A Y S S L L T F H L S T

CCT CGG TCC CAC CAC CTG TAC CAT GCC CGC CTG TGG CTG CAC GTG CTC CCC ACC CTT CCT 135  
P R S H H L Y H A R L W L H V L P T L P

GGC ACT CTT TGC TTG AGG ATC TTC CGA TGG GGA CCA AGG AGG AGG CGC CAA GGG TCC CGC 155  
G T L C L R I F R W G P R R R R Q G S R

ACT CTC CTG GGT GAG CAC CAC ATC ACC AAC CTG GGC TGG CAT ACC TTA ACT CTG CCC TCT 175  
T L L A E H H I T H L G W H T L T L P S

AGT GGC TTG AGG GGT GAG AAG TGT GGT GTC CTG AAA CTG CAA CTA GAC TGC AGA CCC CTA 195  
S G L R G E K S G V L K L Q L D C R P L

GAA GGC AAC AGC ACA GTT ACT GGA CAA CGG AGG CGG CTC TTG GAC ACA GCA GGA CAC CAG 215  
E S N S T V T G Q P R R L L D T A G H Q

CAG CCC TTC CTA GAG CTT AAG ATC CGA GCC AAT GAG CCT GGA GCA GGC CGG GCC AGG AGG 235  
Q P F L E L K I R A N E P G A G R A R R

AGG ACC CCC ACC TGT GAG CCT GCG ACC CCC TTA TGT TGC AGG CGA GAC CAT TAC GTA GAC 255  
R A T P T C E P A T P L C C R R D H Y V D

TTT CAG GAA CTG GGA TGG CGG GAC TGG ATA CTG CAG CCC GAG GGG TAC CAG CTG AAT TAC 275  
F Q E L G W R D W I L C P E G Y Q L N Y

TGC ACT GGG CAG TGC CCT CCC CAC CTG GGT GGC GGC CCA GGC ATT GGT GCC TCT TTC CAT 295  
C I G Q C P F H L A G G F G T A A S F H

TCT GGC GTC TTC AGC CTC CTC AAA GCC AAC AAT CCT TGG CCT GCC AGT ACC TCC TGT TGT 315  
S A V F S L L K A H N P W P A S T S C C

GTC CTT ACT GCC CGA AGG CCC CTC TCT CTC CTC TAC CTG GAT CAT AAT GCC AAT GTG GTC 335  
V P T A R R P L S L L Y L D H N G H V V

AAG ACG GAT GTG CCA GAT ATG GTG GTG GAG GCC TGT GGC TGC AGC TAG 350  
K T D V P D M V V E A C G C S \*

CAA GAG ACC TGG GGC TTT GGA GTG AAG AGC CCA AGA TGA AGT TTC CCA GGC ACA GGG CAT  
CTG TGA CTG GAG GCA TCA GAT TCC TGA TCC ACA CCC CAA CCC AAC AAC CAC CTG GCA ATA  
TGA CTC ACT TGA CCC TAT GGG ACC AAA TGG GCA CTT TCT TGT CTG AGA CTC TGG CTT ATT  
CCA GGT TGG CTG ATG TGT TGG GAG ATG GGT AAA GCG TTT CTT CTA AAG GGG TCT ACC CAG  
AAA GCA TGA TTT CCT GCC CTA AGT CCT GTG AGA AGA TGT CAG GTA CTA GGG AGG GAG GGA  
GGG AAG GTC AGA GAA AAA TTA CTT AGC CTC TCC CAA GAT GAG AAA GTC CTC AAG TGA GGG  
GGA GGA GGA AGC AGA TAG ATG CTC CAG CAG GAC TTG AAG CAG GGT AAG CAG GCT GGC CCA  
GGG TAA GGG CTG TTG AGG TAC CTT AAG GGA AGG TGC AAG AGA TAC CTT TTC CGA TGC CTT  
GTC TTG CAC AGG TGG CCC TGA ACC TGG AGG AGG AGG GGC TCG GCG CTT CTT CAT CTC CGA  
CTT CAC GGA CAC CCC TGA GAT GGT GCC CAG CGA GAG GGA TGG ACC CAG CCT AAG AGA ACG  
TGA GTG CAT GGA TGG GGA GTT GGG GGT COT TAA A

FIG. 5

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Lanes

1. Heart
2. Brain
3. Spleen
4. Lung
5. Liver
6. Skeletal muscle
7. Kidney
8. Testis

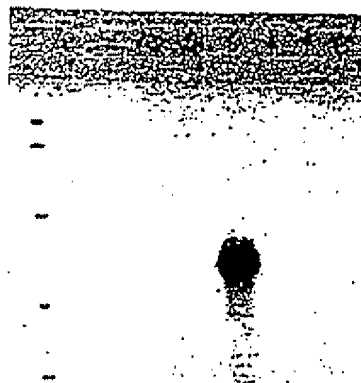
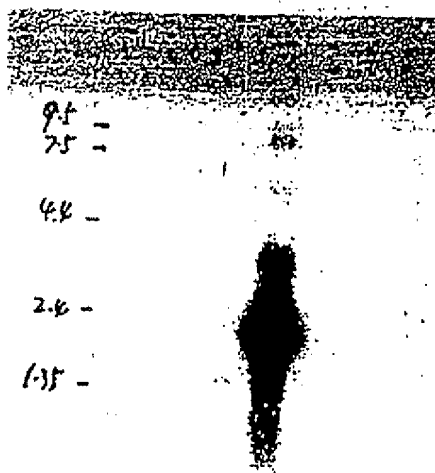


FIG. 6

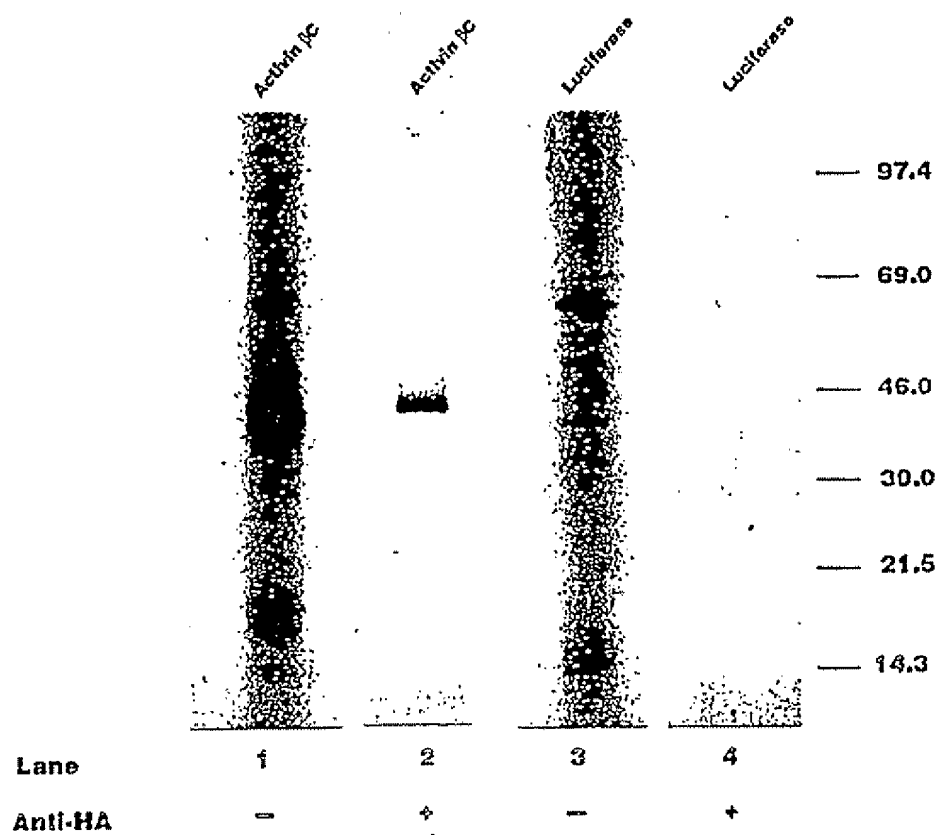


FIG. 7



FIG. 8

**ACTIVIN HEMATOPOIESIS-PROMOTING ACTIVITY**

Condition	Hematopoietic Progenitor Cell Frequency		
	Erythroid	Granulocytic	Magakaryocytic
Control cytokines	32 + 4	76 + 30	23 + 10
Control cytokines + Luciferase	8 + 8	95 + 13	4 + 4
Control cytokines + Mouse Activin $\beta_C$	48 + 12	164 + 4	50 + 16
Increase Over Control	50%	116%	117%
p value (versus Control)	0.09	0.007	0.06
p value (versus Luciferase)	0.008	0.04	0.06

**Total # Hematopoietic Progenitor Cells**

Control Hematopoietic Progenitor Cells	131
Luciferase Hematopoietic Progenitor Cells	107
Activin Hematopoietic Progenitor Cells	262

**FIG. 9**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20882

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04; C12N 15/00, 5/10, 5/16

US CL : 536/23.5; 435/320.1, 325

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG biotech cluster

terms: liver, activin, DNA, nucleic acid, vector, plasmid, hepatocyte, transfect?

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----	FANG, J. et al. Molecular cloning of the mouse activin $\beta_E$ subunit gene. Biochem. Biophys. Res. Comm. 21 November 1996, Vol. 228, No. 3, pages 669-674, see entire document.	1, 3, 5 ----
Y,P ----		5, 6, 8, 10, 11 ----
A,P		2, 7, 9, 13, 14
Y	COCKETT, M. I. et al. The use of engineered E1A genes to transactivate the hCMV-MIE promoter in permanent CHO cell lines. Nuc. Acids Res. 25 January 1991, Vol. 19, No. 2, pages 319-325, see entire document.	5, 6, 8, 10, 12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

04 MAR 1998

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/20882

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WILSON, J. M. et al. A novel mechanism for achieving transgene persistence in vivo after somatic gene transfer into hepatocytes. J. Biol. Chem. 05 June 1992, Vol. 267, No. 16, pages 11483-11489, especially page 11483.	5, 6, 8, 10-12

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/20882

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20882

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-14, drawn to nucleic acid encoding liver activin .beta.E and chimeras thereof, vectors and host cells comprising same.

Group II, claim(s) 15-18 and 25-30, drawn to liver activin protein, chimeras thereof and a method for stimulating cell growth in a vertebrate.

Group III, claim(s) 19-20, drawn to antibodies to liver activin.

Group IV, claim(s) 21-22, drawn to a nucleic acid based method for diagnosing cell growth.

Group V, claim(s) 21 and 23, drawn to a protein expression based method for diagnosing cell growth.

Group VI, claim(s) 24, drawn to a method for diagnosing cell growth by detecting a liver activin mutation.

Group VII, claim(s) 31-36, drawn to a method for inhibiting liver activin expression.

Group VIII, claim(s) 37-38, drawn to a method for inhibiting liver activin signal transduction.

Group IX, claim(s) 39-45, drawn to a method for up-regulating liver activin in vivo.

Group X, claim(s) 46-50, drawn to a method for inducing bone growth.

Group XI, claim(s) 51-55, drawn to a method for stimulating hematopoiesis.

The inventions are distinct, each from the other because of the following reasons: Invention I and inventions IV, VIII, IX, X, XI and XII are related as product and processes of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. In the instant case the nucleic acid of group I can be used for multiple purposes, such as a probes for gene location and detection of mRNA production, as well as the recombinant manufacture of liver activin.

The inventions listed as Groups I-XI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Inventions I, II and III are unrelated. The different inventions are chemically distinct molecules with different building blocks, the nucleic acids of Group I versus the amino acids of Groups II and III, and have different targets, functions and modes of bioactivity. The protein of invention II is a second composition and has no common core structure with invention I. Further, the antibody of invention III is a third composition and shares no common core structure with inventions I or II.

Inventions IV and V are unrelated. In the instant case the different inventions are both drawn to diagnostic assays for cell growth but require different methods and chemically/materially different components for practice.

Inventions VII-XI are unrelated. The different inventions are all drawn to in vivo therapies but have different aims, stimulation versus inhibition, or target tissues, liver, bone or hematopoietic tissue. One of ordinary skill in the art could not anticipate that the same compound would be obvious to use for any given one of the treatments over the use of said compound in any of the other treatments.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.